The Activated Glucocorticoid Receptor Modulates Presumptive Autoregulation of Ribosomal Protein S6 Protein Kinase, p70 S6K*  

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Protein metabolism in eukaryotic organisms is defined by a synthesis-degradation equilibrium that is subject to regulation by hormonal and nutritional signals. In mammalian tissues such as skeletal muscle, glucocorticoids, hormones, specify a catabolic response that influences both protein synthetic and protein degradative pathways. With regard to the former, glucocorticoids attenuate mRNA translation at two levels: translational efficiency, i.e. translation initiation, and translational capacity, i.e. ribosome biogenesis. Glucocorticoids may impair translational capacity through the ribosomal S6 protein kinase (p70 S6K), a recognized glucocorticoid target and an effector of ribosomal protein synthesis. We demonstrate here that the reduction in growth factor-activated p70 S6K activity by glucocorticoids depends upon a functional glucocorticoid receptor (GR) and that the GR is both necessary and sufficient to render p70 S6K subject to glucocorticoid regulation. Furthermore, the DNA binding and transcriptional activation but not repression properties of the GR are indispensable for p70 S6K regulation. Finally, a mutational analysis of the p70 S6K carboxyl terminus indicates that this region confers glucocorticoid sensitivity, and thus glucocorticoids may facilitate autoinhibition of the enzyme ultimately reducing the efficiency with which T389 is phosphorylated.

Glucocorticoids are known to regulate an array of physiological processes including carbohydrate, lipid, and protein metabolism, reproduction and development, activation of the immune system, and cell growth, division, and differentiation. Their mechanism of action involves recognition by the cytosolic, ligand-inducible glucocorticoid receptor (GR),1 which, in the absence of hormone, is inactive by virtue of its interaction with a heat shock protein 90-based chaperone complex. When complexed with its ligand, the GR undergoes a conformational transformation that promotes its subsequent transport into the nucleus. Therein, the GR interacts with discrete glucocorticoid response elements (GREs) within the promoter region of target genes. This mode of action may lead to the hormone-dependent induction or repression of responsive genes, which is further modulated by cell background, GRE context, and accessory transcription factors (1–3).

One protein that is emerging as an important target of glucocorticoid action is the ribosomal protein S6 protein kinase, p70 S6K (4, 5). The S6 kinases are related to other kinases of the AGC (protein kinases A, G, and C) superfamily (reviewed in Ref. 6) and display acute activation in response to numerous stimuli including a broad range of growth factors (reviewed in Ref. 7), integrin-extracellular matrix engagement (8), oxidative (9, 10) and shear stresses (11), phorbol esters (4, 12), transforming oncogenes (12, 13), amino acids (12, 14), protein synthesis inhibitors (12, 15), phosphatase inhibitors (12, 16), and calcium mobilizing agents (17, 18). The activation of p70 S6K derives from a complex series of phosphorylations involving some 13 reported sites (7, 19–23) (see Fig. 1A). These phosphorylations occur in a hierarchical manner commencing with phosphorylation of a cluster of at least four carboxyl-terminal sites localized within the presumptive autoinhibitory pseudosubstrate domain (19). These phosphorylation events have been suggested to transform the conformation of the protein so as to disrupt a putative interaction between the autoinhibitory region and the substrate recognition pocket. As a result, additional internal phosphorylation sites are exposed to activating kinases. Among the internal sites, phosphorylation of Thr229 (21), Ser371 (20), and Thr389 (21) are essential for p70 S6K activation (24). The Thr229 kinase has been identified as 3-phosphoinositide-dependent kinase 1 (PDK1 (24, 26)), PDK2, remains uncharacterized.

p70 S6K serves a critical function in governing the G1-to-S-phase transition, which may in turn, reflect p70 S6K-mediated regulation of ribosome biogenesis. During cell proliferation, an increase in cellular components required for protein synthesis is necessary to support the increase in cell mass that precedes mitosis (reviewed in Refs. 27 and 28). The surge in ribosomal protein mRNA translation that accompanies cell growth may be attributable to a distinctive 5’ polynucleotide sequence designated TOP (terminal oligopyrimidin), which is a feature common to many ribosomal protein-encoding mRNAs (29, 30). This structural element is sufficient to confer mitogen-stimu-
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**Antibodies and Reagents—Anti-p70 S6K (C-18; catalog no. sc-330), which recognizes an epitope within the p70 S6K carboxy terminus, was purchased from Santa Cruz Biotechnology, Inc. Anti-phospho-Thr^{421}/Ser^{424} (catalog no. 9204), which recognizes p70 S6K when doubly phosphorylated on Thr^{421} and Ser^{424}, and anti-phospho-Thr^{389} (catalog no. 9205), which recognizes p70 S6K when phosphorylated on Thr^{389}, were purchased from Cell Signaling Technology. Anti-GR (M-20; catalog no. sc-1004), which recognizes an amino-terminal epitope of GR, was purchased from Santa Cruz Biotechnology, Inc. as was Anti-HA (Y-11; catalog no. sc-805). Purified, recombinant 4E-BP1 (PHAS-I; catalog no. 516675) was purchased from Calbiochem. Dexamethasone sodium phosphate, RU486, IGF-I, and insulin were purchased from Sigma.

*p70 S6K and GR Variants—Amino-terminal hemagglutinin (HA) epitope-tagged rat wild-type (HA-p70 S6K) and D3E (also referred to as D4) p70 S6K constructs have been described previously (35). The D3E S429D variant was created using a PCR-based site-directed mutagenesis strategy (QuickChange site-directed mutagenesis kit, Stratagene) in a previously (38, 39). For each immunoblot presented, samples from each construct were blotted onto nitrocellulose, washed, immunostained with anti-GR, and destained and reblotted onto nitrocellulose, washed, immunostained with anti-p70 S6K. This procedure has been detailed elsewhere (5).

**A**

**B**

![Diagram](https://example.com/diagram.png)

**Fig. 1. Regulatory components of p70 S6K and GR polypeptides.** A, a generalized diagrammatic representation of the p70 S6K protein is presented emphasizing four modular domains: the amino terminus (NT), the catalytic activation loop (CAT), the linker region, and the autoinhibitory pseudosubstrate domain, which is localized to a portion of the carboxyl terminus (CT). Also displayed are the relative positions of reported phosphorylation sites. The phosphorylation sites examined in this study, Thr^{389}, Thr^{421}, and Ser^{424}, are highlighted. The relative position of the truncation in the ΔCT104 variant is also presented. B, a schematic illustration of the full-length rat GRs molecule is depicted accenting important features: (i) AF-1 resides within the amino-terminal segment of the GR and represents a ligand-independent transcriptional enhancement domain; (ii) the DNA-binding domain (DBD) is localized centrally and harbors residues important for DNA recognition; (iii) the LBD is situated within the carboxyl terminus of the GR, overlapping the ligand-binding domain, and the autoinhibitory pseudosubstrate domain, which is localized to a portion of the carboxyl terminus (CT). Also displayed are the relative positions of reported phosphorylation sites. The phosphorylation sites examined in this study, Thr^{389}, Thr^{421}, and Ser^{424}, are highlighted. The relative position of the truncation in the ΔCT104 variant is also presented. B, a schematic illustration of the full-length rat GRs molecule is depicted accenting important features: (i) AF-1 resides within the amino-terminal segment of the GR and represents a ligand-independent transcriptional enhancement domain; (ii) the DNA-binding domain (DBD) is localized centrally and harbors residues important for DNA recognition; (iii) the LBD is situated within the carboxyl terminus of the GR, and contains residues critical for ligand recognition. AF-2, a ligand-independent transcriptional regulatory domain, also resides within the carboxyl terminus of the GR, overlapping the ligand-binding domain. The relative positions of truncation for 407C and N525 GR variants are presented also.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfection—COS7 and HEK293 cells (Dr. Anthony Pegg, The Pennsylvania State University College of Medicine) and spontaneously differentiating rat L6 and mouse C2C12 skeletal myoblasts (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin. Cells were seeded in 6-well plates at 5 × 10^4 cells/well and cultured in the presence of serum for 24 h. The culture medium was then replaced with serum-free Dulbecco’s modified Eagle’s medium for an additional 24 h, and cells were assayed subsequently.

Chinese hamster ovary cells stably overexpressing the human insulin receptor (CHO-IR; Joseph Avruch, Harvard Medical School) were cultured in Ham’s F12 medium supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin. CHO-IR cells were transiently cotransfected with 1 μg each of plasmid DNA encoding p70 S6K and GR variants as indicated in the legend of relevant figures using LipofectAMINE 2000 (Invitrogen) as suggested by the manufacturer. At 24 h post-transfection, cells were rinsed with phosphate-buffered saline and cultured in serum-free Ham’s F12 medium for an additional 24 h in the presence or absence of dexamethasone as indicated. Cells were subsequently stimulated for 30 min with insulin and then assayed.

**Western Blot Analysis—Cellular protein was prepared initially by harvesting cells in buffer A (50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 100 μM phenylmethylsulfonyl fluoride, 200 μM benzamidine, 800 μM leupeptin, 1 mg/ml pepstatin, 1 mM Na_{2}VO_{4}). Solubilization of cell membranes was facilitated by rockering an orbital rocker for an additional 20 min. Cell lysates were then clarified by centrifugation at 14,000 × g for 20 min at 4 °C and subjected to Western analysis as described previously (38, 39). For each immunoblot presented, samples from each condition were pooled from either duplicate or triplicate assays prior to electrophoresis. Thus, the depicted Western blots accurately reflect mean unmagnocyt activity given per experiment.

**p70 S6K Activity—**This procedure has been detailed elsewhere (5).

Briefly, the activity of endogenous or ectopically expressed p70 S6K was determined in anti-p70 S6K or anti-HA immune complexes, respectively, by measuring 32P incorporation into a synthetic peptide (AKRRRLSSLRA) derived from the region of S6 phosphorylated naturally. Because, in the cell system employed here, the expression levels of different p70 S6K constructs was somewhat variable between treatment conditions, when p70 S6K was expressed ectopically, kinase activity determinations were normalized for protein expression levels.
Experimental Procedures.

For p70 S6K activity as described under "Experimental Procedures." A, cells were incubated in the absence or presence of dexamethasone (1 μM) for 4 h and subsequently stimulated with insulin (100 nM), IGF-I (20 ng/ml), or serum (10%, v/v) for 30 min as indicated. Following stimulation, cells were lysed, normalized for protein content, and then incubated with anti-p70 S6K antibody to isolate p70 S6K immune complexes. The immunoprecipitates were then assayed for p70 S6K activity as described under "Experimental Procedures." The condition depicts combined data obtained from two independent experiments each of which was performed in duplicate. The data are expressed as the means ± S.E. for each condition within an experiment wherein the activity obtained after serum stimulation is arbitrarily defined as 100%. B, each of the four cell lines examined was deprived of serum for 24 h prior to the addition of dexamethasone (1 μM) for an additional 4 or 24 h as indicated. Cell extracts obtained after the various treatments were normalized for protein content and subjected to Western analysis using anti-p70 S6K and anti-GR antibodies as detailed under "Experimental Procedures." The results are typical for at least three independent determinations.

**RESULTS**

Although glucocorticoid hormones affect a number of biological processes, often the control of discrete physiologic events is observed within a defined subset of glucocorticoid-sensitive cell types. Indeed, the regulation of p70 S6K by glucocorticoids has been demonstrated in skeletal muscle (40) and in interleukin-2-dependent T lymphocytes (4). However, in a previous study, p70 S6K activity in NIH3T3 fibroblasts was shown to be completely insensitive to glucocorticoid treatment (4) suggesting that, in this particular cell line, some component essential for glucocorticoid action is lacking. In contrast, in cultures of undifferentiated myoblasts, glucocorticoids repress p70 S6K activation (5). We rationalized that the relative glucocorticoid sensitivities observed in various cell types may reflect the presence or absence of the GR. In the present study, the ability of glucocorticoids to repress p70 S6K activity was examined in four cell lines. Two lines were found to be completely insensitive to glucocorticoids insofar as p70 S6K regulation was concerned (COS7 and HEK293), whereas C2C12 and L6 myoblasts exhibited mild (~10% inhibition, C2C12) to moderate (35–50% inhibition, L6) sensitivities (Fig. 2A). Interestingly, analysis of unstimulated cell extracts prepared from each of the four cell types revealed that the responsiveness of p70 S6K to glucocorticoids was not only related to the presence of the GR but perhaps also to the activity of the receptor, as indicated by the greater extent of GR down-regulation observed in L6 versus C2C12 cells (cf. Fig. 2, A and B). The absence of endogenous GR immunoreactivity observed in COS7 (monkey) and HEK293 (human) lysates (Fig. 2B, lanes 1–3 and 4–6, respectively) was not because of an inability of the antibody to recognize the GR from different species, as it displays broad cross-reactivity with GR homologs from rat to human (41). Whereas no change in p70 S6K content was observed following 4 h of hormone treatment, it did appear that in C2C12 and L6 cells exposed to glucocorticoid for 24 h, the levels of p70 S6K protein were diminished (Fig. 2B, lanes 9–12). Nevertheless, in C2C12 and L6 cells treated with glucocorticoid, the multiple electrophoretic forms of p70 S6K redistributed to forms of higher mobility (Fig. 2B) indicating that p70 S6K was dephosphorylated. Such an effect was not observed in COS7 or HEK293 cells. Whereas in L6 cells expression of GR was down-regulated following administration of dexamethasone, GR expression was only minimally affected in C2C12 cells (Fig. 2B). The reduction in GR protein was related temporally (Fig. 2B) and has been shown to occur at both the level of gene transcription (42, 43) and post-translationally (44). Hence, the relative sensitivity of p70 S6K to regulation by glucocorticoids is related to the availability of the GR, presumably to mediate this regulation. Surprisingly, however, GR regulation of p70 S6K appears to be stronger in L6 cells compared with C2C12 cells, despite relatively less endogenous receptor.

The primary mode of glucocorticoid action entails recognition of the hormone by the GR, which converts the receptor into an activated transcriptional modulator. The partial GR agonist RU486 (also called mifepristone) competes with natural and synthetic glucocorticoids for binding to the GR. The resultant RU486-ligated GR is efficiently imported into the nucleus but manifests a generalized defect in transcriptional activation. Nevertheless, this form of activated GR remains sufficient for transcriptional repression in certain cell systems and promoter contexts (45–47). Dexamethasone, a pure glucocorticoid agonist, inhibited IGF-I-stimulated activation of p70 S6K in a dose-related fashion within 4 h of exposure to the steroid (Fig. 3A) with 50% inhibition achieved at a concentration of 1 μM. To

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**Fig. 2.** Regulation of p70 S6K by glucocorticoids is conditionally cell-selective. Quiescent COS7, HEK293, C2C12, and L6 cells were cultured as described under “Experimental Procedures.” A, cells were incubated in the absence or presence of dexamethasone (1 μM) for 4 h and subsequently stimulated with insulin (100 nM), IGF-I (20 ng/ml), or serum (10%, v/v) for 30 min as indicated. Following stimulation, cells were lysed, normalized for protein content, and then incubated with anti-p70 S6K antibody to isolate p70 S6K immune complexes. The immunoprecipitates were then assayed for p70 S6K activity as described under "Experimental Procedures." The condition depicts combined data obtained from two independent experiments each of which was performed in duplicate. The data are expressed as the means ± S.E. for each condition within an experiment wherein the activity obtained after serum stimulation is arbitrarily defined as 100%. B, each of the four cell lines examined was deprived of serum for 24 h prior to the addition of dexamethasone (1 μM) for an additional 4 or 24 h as indicated. Cell extracts obtained after the various treatments were normalized for protein content and subjected to Western analysis using anti-p70 S6K and anti-GR antibodies as detailed under "Experimental Procedures." The results are typical for at least three independent determinations.
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There are two independent experiments performed in triplicate.

Representative of two independent experiments performed in triplicate. The addition of dexamethasone, cells were stimulated with IGF-I (20 ng/ml) for 45 min prior to addition of dexamethasone (1 μM) for 30 min. Cells were subsequently assayed for p70 S6K activity as described in the legend for Fig. 2. B, cells were pretreated with RU486 (10 μM) for 45 min prior to addition of dexamethasone (1 μM); 4 h after the addition of dexamethasone, cells were stimulated with IGF-I (20 ng/ml) and then assayed for p70 S6K activity. The data are expressed as the means ± S.E. for each condition within an experiment. The data are representative of two independent experiments performed in triplicate.

Fig. 3. Antagonism of the GR interferes with regulation of p70 S6K by glucocorticoids. A, serum-deprived L6 cells were pretreated for 4 h with the GR agonist dexamethasone or the partial GR agonist RU486 at the doses indicated prior to stimulation with IGF-I (20 ng/ml) for 30 min. Cells were subsequently assayed for p70 S6K activity as described in the legend for Fig. 2. B, cells were pretreated with RU486 (10 μM) for 45 min prior to addition of dexamethasone (1 μM); 4 h after the addition of dexamethasone, cells were stimulated with IGF-I (20 ng/ml) and then assayed for p70 S6K activity. The data are expressed as the means ± S.E. for each condition within an experiment. The data are representative of two independent experiments performed in triplicate.

The deletion removes the ligand binding domain (LBD) and activation function 2 (AF-2), an agonist-dependent core transcriptional activation domain. Furthermore, the absence of the LBD renders N525 constitutively active with regard to transcriptional activation mediated by AF-1, an amino-terminal, hormone-independent transcriptional enhancement function (36). Interestingly, N525 failed to inhibit insulin-stimulated p70 S6K activity implicating the involvement of AF-2 in GR-mediated control of p70 S6K (Fig. 5, lanes 5 and 6). To evaluate the sufficiency of AF-2 to confer p70 S6K regulation, a receptor mutant comprising the carboxyl-terminal 407 amino acids, which bears the LBD and AF-2, failed to inhibit glucocorticoid-induced regulation of p70 S6K (Fig. 5, lanes 7 and 8). Collectively, the inability of either N525 or 407C to regulate p70 S6K suggests that neither the carboxyl nor amino terminus alone is sufficient for such regulation, although functions displayed in both regions are required. A GR point mutant defective in DNA binding, R466K, was similarly nonfunctional, underscoring the necessity of physical GR-DNA contacts in this process (Fig. 5, lanes 11 and 12). Finally, the K461A GR variant was similar to the wild-type GR with regard to inhibition of p70 S6K (Fig. 5, lanes 14 and 15). It is believed that the Lys → Ala substitution at position 461 “locks” the receptor in a purely activating conformation. As such, K461A enhances transcription at all promoters including exogenously expressed p70 S6K are robustly activated in these cells when treated with low nanomolar concentrations of insulin (Ref. 48 and Fig. 4A), an effect that is completely inhibited by pretreatment of the cells with rapamycin. The CHO-IR cells were cotransfected with the full-length, wild-type GR and a p70 S6K variant that bears an amino-terminal HA epitope tag. In this coexpression system, the endogenous pool of GR was clearly insufficient to modulate ectopically expressed p70 S6K (Fig. 4B) as no effect on p70 S6K activity was observed in dexamethasone-treated cells transfected with empty vector. Alternatively, when overexpressed, ectopic GR inhibited p70 S6K activity to an extent similar to that observed in L6 myoblasts and did so in a dexamethasone-dependent manner (cf. Fig. 4, B and C). In both the natural (L6) and ectopic expression (CHO-IR) systems utilized in this study, the extent of inhibition of p70 S6K by glucocorticoids ranged reproducibly from 35 to 50%. Moreover, this regulation was strictly dependent on the presence of glucocorticoid. As mentioned above, prolonged activation of the GR leads to its homologous down-regulation both through transcriptional and post-translational mechanisms (49). This phenomenon would explain the glucocorticoid-dependent regulation of GR protein observed under most conditions utilized in this study (e.g. Fig. 4C).

Monfar and Blenis (4) have demonstrated that the glucocorticoid-induced inhibition of interleukin-2-stimulated p70 S6K activity is abrogated in T cells administered the transcriptional inhibitor actinomycin D. Furthermore, we have previously demonstrated the sufficiency of actinomycin D to prevent the glucocorticoid-induced dephosphorylation of p70 S6K in L6 myoblasts (16). These reports suggest that p70 S6K is regulated by glucocorticoids in a transcriptionally dependent manner. To explore the transcriptional properties of the GR essential for p70 S6K inhibition, a series of GR variants was individually cotransfected with HA-p70 S6K into the CHO-IR cells (Fig. 5). The consequences of glucocorticoid-dependent activation of the individual mutants was examined subsequently by immunoprecipitation of the exogenously expressed kinase using an antibody to the HA epitope present at the amino terminus of the expressed protein. The N525 GR variant displays a carboxyl-terminal truncation resulting in omission of amino acids 526–795 of the full-length rat GR (see Fig. 1B). The deletion removes the ligand binding domain (LBD) and activation function 2 (AF-2), an agonist-dependent core transcriptional activation domain. Furthermore, the absence of the LBD renders N525 constitutively active with regard to transcriptional activation mediated by AF-1, an amino-terminal, hormone-independent transcriptional enhancement function (36). Interestingly, N525 failed to inhibit insulin-stimulated p70 S6K activity implicating the involvement of AF-2 in GR-mediated control of p70 S6K (Fig. 5, lanes 5 and 6). To evaluate the sufficiency of AF-2 to confer p70 S6K regulation, a receptor mutant comprising the carboxyl-terminal 407 amino acids, i.e. amino acids 389–795 (407C), was coexpressed with p70 S6K. Clearly, 407C, which bears the LBD and AF-2, failed to inhibit p70 S6K (Fig. 5, lanes 8 and 9). Collectively, the inability of either N525 or 407C to regulate p70 S6K suggests that neither the carboxyl nor amino terminus alone is sufficient for such regulation, although functions displayed in both regions are required. A GR point mutant defective in DNA binding, R466K, was similarly nonfunctional, underscoring the necessity of physical GR-DNA contacts in this process (Fig. 5, lanes 11 and 12). Finally, the K461A GR variant was similar to the wild-type GR with regard to inhibition of p70 S6K (Fig. 5, lanes 14 and 15). It is believed that the Lys → Ala substitution at position 461 “locks” the receptor in a purely activating conformation. As such, K461A enhances transcription at all promoters including
those in which the wild-type GR represses (3). Collectively, these data argue strongly in favor of a mode of action involving transcriptional activation rather than repression. In all cases, the degree of inhibition correlates positively with the extent of p70 S6K dephosphorylation at T389 (Fig. 5, lanes 3 and 15).

We have previously demonstrated that the attenuation of p70 S6K activation by glucocorticoids correlates positively with a selective dephosphorylation of sites residing within the carboxyl-terminal pseudosubstrate and putative autoinhibitory region (5). Specifically, in cells stimulated with IGF-I, Thr421, and Ser424 are subject to dephosphorylation, whereas the phosphorylation of Ser411 persists. Additionally, Thr389 is dephosphorylated in glucocorticoid-treated cells. These findings support one of two mutually exclusive possibilities: (i) glucocorticoid action affects both Thr421/Ser424 and Thr389 distinctly and individually (e.g. via interference of their respective

FIG. 5. Inhibition of p70 S6K by glucocorticoids requires the DNA binding and transcriptional activation properties of the GR. CHO-IR cells were transfected with plasmids encoding HA-p70 S6K and wild-type GR and one of four GR variants: (i) a carboxyl-terminally truncated construct (N525) bearing only amino acids 1–525; the LBD and AF-2 are omitted in this construct; (ii) an amino-terminal truncation mutant (R466K); or (iv) a GR variant defective in repression (K461A). Transient transfectants were deprived of serum and incubated with or without dexamethasone (1 μM) for 24 h. Cells were subsequently stimulated for 30 min with insulin (10 nM) and harvested. 90% of the cell extract was utilized for the determination of p70 S6K activity as described in the legend for Fig. 4A, whereas the remaining 10% was subjected to Western analysis. Comparable levels of GR expression were verified by immunoblotting (data not shown). The data are expressed as means ± S.E. within an experiment and are representative of at least three independent determinations performed in triplicate. Anti-phospho-T389-p70 S6K and anti-HA immunoblots are shown below the graph.
Fig. 6. p70 S6K inhibition by glucocorticoids involves regulation at both the autoinhibitory pseudosubstrate domain of the kinase and at Thr389. CHO-IR cells were co-transfected with expression plasmids encoding the wild-type GR in addition to HA-p70 S6K or one of four p70 S6K mutants: (i) a carboxyl-terminal truncation variant in which amino acids 399–502, which harbors the pseudosubstrate autoinhibitory region, have been deleted (ΔCT104); (ii) a quadruple point mutant in which Ser411, Ser418, Thr421, and Ser424 have been substituted with the corresponding acidic amino acids (D3E); (iii) D3E with an additional acidic substitution at Ser429, or (iv) a variant bearing a phosphomimic mutation at Thr389 (T389E). Transient transfectants were deprived of serum and incubated with or without dexamethasone (1 μM) for 24 h. Cells were subsequently stimulated for 30 min with insulin (10 nM) and harvested. 90% of the cell extract was utilized for determination of p70 S6K activity as described in the legend for Fig. 4A, and the remaining 10% was subjected to Western analysis. The data are expressed as means ± S.E. within an experiment and are representative of at least three independent determinations performed in triplicate. The potential degradation of D3E and D3E S429D constructs was noted (lanes 5–8).

In this report, we demonstrate that the ability of glucocorticoids to regulate p70 S6K departs not only upon a functional GR but also upon cell context. Results derived from both pharmacological and mutational analyses indicate that glucocorticoid-induced control of p70 S6K relies upon DNA-dependent transcriptional enhancement by the GR, consistent with the transcriptional dependence of this effect demonstrated previously (4). By inference, GR activation leads to the induction of one or more regulatory gene products that, individually or collectively, interfere with activation of p70 S6K. This interference of p70 S6K activation manifests itself in the form of dephosphorylation of presumed autoinhibitory sites in the p70 S6K carboxyl-terminal tail, which secondarily results in the inefficient phosphorylation or dephosphorylation of Thr389.

DISCUSSION

In this report, we demonstrate that the ability of glucocorticoids to regulate p70 S6K depends not only upon a functional GR but also upon cell context. Results derived from both pharmacological and mutational analyses indicate that glucocorticoid-induced control of p70 S6K relies upon DNA-dependent transcriptional enhancement by the GR, consistent with the transcriptional dependence of this effect demonstrated previously (4). By inference, GR activation leads to the induction of one or more regulatory gene products that, individually or collectively, interfere with activation of p70 S6K. This interference of p70 S6K activation manifests itself in the form of dephosphorylation of presumed autoinhibitory sites in the p70 S6K carboxyl-terminal tail, which secondarily results in the inefficient phosphorylation or dephosphorylation of Thr389.

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Note: The text is a partial transcription of the content from the image. It includes the main points and context of the discussion related to the regulation of p70 S6K by glucocorticoids.
Transcriptional Scenarios Involved in Regulation of p70 S6K by Glucocorticoids—A hallmark of transcriptional control by nuclear receptors is a temporal lag between the initial exposure to hormone and the resultant biologic outcome. The delay is due to the time requirement for significant modulation of relevant target protein levels necessary to elicit the desired cellular response. With regard to the GR, its activation and movement into the nucleus is appreciable within 10 min (51) suggesting that the expression of primary GR targets may be altered rapidly. In contrast, other consequences of glucocorticoid action may not manifest until several rounds of transcriptional induction are allowed and thus may not evolve for several hours or days. For example, in fIIE cells stably transfected with the GR, glucocorticoids augment the expression of the IxBa protein within 2.5 h (45), whereas in HeLa cells, 8 h of glucocorticoid treatment is required to enhance expression of the p57Kip2 protein (52). Interestingly, in glucocorticoid-sensitive systems, the activity of p70 S6K is down-regulated within the first hour of hormone treatment independent of a change in the relative amount of its protein (4, 16), implicating acute regulation of the genome.

How, precisely, does the GR achieve such transcriptional control? The GR exerts its positive and negative transcriptional regulatory functions through specific DNA elements. At classical GREs, the agonist-bound GR binds directly to DNA and recruits distinct AF-1-specific (53) and AF-2-specific (54-57) coactivators through a mechanism that is antagonized by RU486. Furthermore, these elements are effectively activated by the K461A mutant (3) but not by the DNA binding mutant, R466K (37). The relative contributions of AF-1 and AF-2 at these GREs are often variable, and in some instances both functions are required (58). This is exactly the pattern observed for GR inhibition of p70 S6K.

Transcriptional repression can occur through three classes of negative GREs: composite (59), tethering (60, 61), and occluding nGREs (62, 63). At composite and tethering nGREs, the GR represses transcription through contacts with DNA and other adjacent bound factors (composite) or exclusively via protein-protein interactions (tethering). In these cases, RU486 is usually (64-66) but not always (60) able to induce repression, whereas the K461A mutant invariably fails to repress (3). This finding is indirect contrast to the results obtained in the present study for the GR effects on p70 S6K. Finally, at occluding nGREs, the GR is thought to bind specifically to DNA, and in doing so, competitively displace essential transcription factors such as TATA-binding protein (63, 67). For this class of nGREs, K461A but not R466K represses transcription (37, 63). At these sites however, the DNA binding domain of the GR alone is
sufficient for repression (63), and RU486 is usually active (62). This spectrum of properties is also inconsistent with the data presented herein regarding GR-mediated inhibition of p70 S6K. Taken together, these results suggest that inhibition of p70 S6K activity by glucocorticoids occurs through the transcriptional induction of one or more factors that directly or indirectly negatively affect this kinase rather than through the repression of positively acting factors.

The Autoregulatory Carboxyl-terminal Pseudosubstrate Domain of p70 S6K Is the Primary Site of Glucocorticoid Action—With the crystal structure of p70 S6K not yet solved, our current understanding of p70 S6K activation derives from extensive mutational and structure/function analyses, thus relying on inferences rather than direct crystallographic information. Nevertheless, the findings of these numerous studies have afforded a hypothetical framework for the molecular basis of p70 S6K activation. In the absence of mitogen, p70 S6K is maintained quiescent by virtue of a putative intramolecular interaction between the carboxyl-terminal pseudosubstrate domain and the catalytic pocket. Two key observations support this premise. First, the primary amino acid sequence surrounding the cluster of p70 S6K carboxyl-terminal phosphorylation sites exhibits strong homology to the region within its substrate, S6, that it phosphorylates (19, 68); hence, the designation “pseudosubstrate” domain. Furthermore, incubation of p70 S6K with synthetic peptides derived from this homologous region substantially hinders its activation (68, 69) demonstrating that this module, in the context of the full-length p70 S6K polypeptide, may serve an autoinhibitory function. Stimulation of cells in culture with growth factors initiates signals that lead to the hierarchical activation of p70 S6K, commencing with the (Ser/Thr)Pro-specific phosphorylation of a cluster of 4–6 residues with the carboxyl terminus of the kinase (19). This battery of phosphorylations promotes a conformational change that relieves autoinhibition imposed by the carboxyl terminus and, in addition, exposes additional phosphorylation sites initially buried within interior of p70 S6K. Ultimately, it is the phosphorylation of these internal sites, namely Thr229, Ser371, and Thr389, that synergistically imparts optimal kinase activity.

A hypothesis to explain the inactivation of p70 S6K observed following exposure of cells to glucocorticoids is that, under such conditions, the carboxyl-terminal cluster of phosphorylation sites is the predominant site of dephosphorylation. As a consequence, glucocorticoid treatment promotes the autoinhibited conformation of p70 S6K, which in turn interferes with the phosphorylation of additional, internally situated, activating phosphorylation sites such as Thr389. This model is supported by several observations. (i) The D3E quadruple point mutant, bearing phosphomimetic substitutions at four carboxyl-terminal (Ser/Thr)Pro sites, is partially resistant to inhibition by glucocorticoids. Further acidic substitution of Ser249 in the D3E context slightly enhances this resistance. (ii) Omission of the entire carboxyl terminus rescues the resultant p70 S6K variant from inactivation by glucocorticoids. Moreover, insulin-stimulated phosphorylation of Thr389 is preserved in this mutant. (iii) Finally, the T389E construct, which displays partial rapamycin insensitivity (21), is totally glucocorticoid-resistant despite dephosphorylation of carboxyl-terminal sites similar in extent to that observed in the parental wild-type construct. The lack of total rescue from glucocorticoid-induced inhibition in D3E S429D implies that the single unmutated site within the carboxyl-terminal tail, i.e. at Thr447, may remain subject to dephosphorylation and as such may allow partial activation of the kinase. Alternatively, the acidic mutations in D3E and D3E S429D may not completely recapitulate natural phosphorylation and, hence, the function of the cluster of sites within the p70 S6K tail. A third possibility is that glucocorticoid action regulates p70 S6K via both autoregulatory site dephosphorylation as well as via some additional cryptic input mediated by the carboxyl terminus. Nevertheless, the data presented herein argue in favor of glucocorticoid control of p70 S6K occurring through the carboxyl-terminal pseudosubstrate and putative autoinhibitory region.

The Implications of GR Regulation of Translational Control Pathways—Among the first biologic functions ascribed to glucocorticoid hormones was the potent modulation of protein metabolism. As is starkly manifest in Cushing’s syndrome, an endocrinopathic anomaly characterized by glucocorticoid overproduction, glucocorticoid excess is associated with atrophy of the skeletal musculature and derives from both the acceleration of protein degradation (70–72) and attenuation of mRNA translation rates (73–76). In vivo, 4 h of exposure to glucocorticoids is sufficient to reduce p70 S6K activation, hinder assembly of the eukaryotic translation initiation factor 4F complex, and suppress global protein synthesis in skeletal muscle (40), suggesting that glucocorticoids acutely and negatively affect protein biosynthesis at the level of translation initiation. Consistent with this idea, actively engaged polysomes disassociate in skeletal muscle following exposure to glucocorticoids (74, 77, 78), indicating that the initiation phase of mRNA translation is impaired. Moreover, prolonged exposure to glucocorticoids is associated with a reduction in both mRNA translation rates and total cellular RNA (74, 77); thus, glucocorticoid action, over an extended period, hinders ribosome biogenesis. Indeed, the translation of mRNAs encoding ribosomal proteins is markedly attenuated in glucocorticoid-treated cells (79). Furthermore, in humans, a 6-h infusion of cortisol reduces the number of ribosomes by 30% in the skeletal musculature (80). Collectively, glucocorticoid control of protein synthesis is biphasic: (i) in the short term, translational efficiency is affected through attenuation of initiation factor activities; and (ii) in the long term, translational capacity is reduced as a result of diminished biosynthesis of ribosomal and ancillary translational components. Whether glucocorticoids elicit both short- and long-term effects through the same mechanism(s) is an intriguing question to be addressed in future studies.

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