Mechanisms of regulation of $G_{11\alpha}$ protein by dexamethasone in osteoblastic UMR 106–01 cells

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Cheung, Ricky, and Jane Mitchell. Mechanisms of regulation of $G_{11\alpha}$ protein by dexamethasone in osteoblastic UMR 106–01 cells. Am J Physiol Endocrinol Metab 282: E24–E30, 2002.—We have previously demonstrated that glucocorticoids increased $G_{q/11\alpha}$ protein expression and phospholipase C activity in the rat osteosarcoma cell line UMR 106–01. In this study, we demonstrated that $G_{11\alpha}$ is the primary $G_q$-subtype family member expressed in UMR cells. Dexamethasone treatment increased the expression of $G_{11\alpha}$ protein in both a time- and a dose-dependent manner. Glucocorticoid treatment significantly increased the half-life of $G_{11\alpha}$ protein from 20.3 to 63 h. Steady-state $G_{11\alpha}$ mRNA level was also increased by glucocorticoid treatment by ~70%. This change was not the result of changes in RNA stability but rather the result of increased transcription, because the glucocorticoid-mediated upregulation of $G_{11\alpha}$ mRNA was blocked by the transcription inhibitor actinomycin D. The dexamethasone induction of $G_{11\alpha}$ mRNA occurred after a time lag of 12–24 h and was blocked by the protein synthesis inhibitor cycloheximide. These results suggest that the dexamethasone-induced rise in $G_{11\alpha}$ protein results primarily from changes in the degradation rate of the protein, whereas changes in $G_{11\alpha}$ mRNA play a smaller role and require de novo synthesis of regulatory protein(s).

Glucocorticoids; G protein; $G_{11\alpha}$; osteosarcoma

HETEROTRIMERIC G PROTEINS are a family of guanine nucleotide-binding proteins composed of three subunits: $\alpha$, $\beta$, and $\gamma$. Because G proteins are crucial for a large array of transmembrane signaling (7), any alteration in protein levels of these three subunits in the cell could potentially affect its ability to respond to external signals. In addition, both the amount and the type of G proteins expressed in individual cells may change the overall cellular response to external stimuli. Very little is known, however, about how the cellular levels of each G protein subtype are regulated to allow the G proteins to fulfill the needs of particular cells. In vivo, the status of various hormones has been reported to affect the steady-state levels of several G protein subunits. Hypothyroidism has been reported to decrease the steady-state level of $G_{\alpha}$ in reticulocytes (28) but to increase the steady-state levels of $G_{\alpha}$ (12), $G_{\alpha}$ (13), and $G_{\beta/\gamma}$ (21) in adipose tissue. Subsequently, $G_{\beta/\gamma}$ expression has been shown to be regulated at the mRNA level (19). Therefore, G protein subunits are differentially regulated by thyroid hormones in fat cells at both the protein and mRNA levels. Glucocorticoids have also been shown to modulate the steady-state levels of various G proteins. Adrenalectomy increases the amount of $G_{\alpha}$ while decreasing the level of $G_{\alpha}$ in liver (6) and $G_{\beta/\gamma}$ in adipose tissue (22). Alteration in the mRNA level of $G_{\alpha}$, $G_{\alpha}$ (24), and $G_{\beta/\gamma}$ (23) has subsequently been shown to be responsible for the changes in the steady-state level of G protein subunits after adrenalectomy. Glucocorticoid administration increases the protein and mRNA levels of $G_{\alpha}$ but decreases those of $G_{\alpha}$ in rat cerebral cortex (24). In vitro treatment with dexamethasone, a synthetic glucocorticoid analog, has also been shown to increase the protein and mRNA levels of $G_{\alpha}$ in rat pituitary cells (3) and $G_{\beta/\gamma}$ in rat fat cells (23). Therefore, the biochemical mechanism underlying the change in G protein levels by glucocorticoids may be altered transcription of mRNA encoding the G protein subunits.

Most of the work performed to date has examined the effect of hormone status on regulation of components of the adenyl cyclase system. To understand the effect of glucocorticoids on the phospholipase C (PLC) system, our laboratory has investigated the effect of dexamethasone on the expression of $G_{q/11\alpha}$ protein and the hormone stimulation of PLC activity in rat bone cells. We examined the effect of glucocorticoids on parathyroid hormone (PTH) activation of two signal transduction pathways: PLC and adenyl cyclase (AC), in rat osteosarcoma cells UMR 106–01 and found that glucocorticoids are more potent regulators of the PLC pathway than of the AC pathway in UMR cells (16). Dexamethasone increased PTH-activated PLC activity correlated with an elevated $G_{q/11\alpha}$ protein expression. Glucocorticoid administration to rats has also been reported to increase $G_{q/11\alpha}$ levels and vasopressin-stimulated PLC activity in the pituitary (18). In an effort to gain better insight into the molecular mechanism by which glucocorticoids regulate the $G_q$-mediated signaling system, we examined the effects of dexamethasone treatment on the steady-state expression and stability of $G_{q/11\alpha}$ protein and mRNA.

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EXPERIMENTAL PROCEDURES

Materials. UMR-106–01 rat osteosarcoma cells were a kind gift from Dr. N. C. Partridge, St. Louis University School of Medicine (St. Louis, MO). Dexamethasone and Tran-[35]S)methionine (1,000 Ci/mmol) were purchased from ICN Biochemicals (Aurora, OH). Pansorbin was purchased from Calbiochem (San Diego, CA). Recombinant G11α protein was purchased from Chemicon (Temecula, CA). Antibodies raised to peptides corresponding to the amino acid sequence within the amino-terminal domain of Gα11 and G11α, or to the carboxy terminus of Gα11α, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Full-length mouse G11α cDNA was generously provided by Dr. M. I. Simon, California Institute of Technology Division of Biology (Pasadena, CA). [α-32P]dCTP (3,000 Ci/mmol) was purchased from Perkin-Elmer (Markham, ON). TRIZol Reagent, M-MLV reverse transcriptase, and all tissue culture media were from GIBCO-BRL Life Technologies (Burlington, ON). Actinomycin D was purchased from Biomol (Plymouth Meeting, PA). Cycloheximide and 5,6-dichlorobenzimidazole riboside (DRB) were purchased from Sigma (Oakville, ON).

Cell culture. UMR-106–01 cells were maintained in DMEM-Ham’s F-12 (50:50) supplemented with 5% fetal bovine serum (FBS), 1 U/ml penicillin, and 1 µg/ml streptomycin and grown at 37°C in a humidified 95% air-5% CO2 atmosphere. Cells were treated with dexamethasone or vehicle (0.001% ethanol) in medium containing 5% FBS, with replacement of medium every 24 h.

Western blot analysis. After treatment with dexamethasone or vehicle, cells were harvested from 75-cm2 flasks using 0.1% trypsin and resuspended in a lysis buffer containing 20 mM Tris (pH 7.5), 1 mM EGTA, and 1 mM dithiothreitol. Cells were homogenized, and protein concentrations were determined by amido black protein assay (25). For Western immunoblotting analysis, 20, 40, and 60 µg of protein were run on 11% acrylamide gels by use of the Laemmli method and 0.1% trypsin and resuspended in a lysis buffer containing 20 mM Tris (pH 7.5), 1 mM EGTA, and 1 mM dithiothreitol (0.001% ethanol) in medium containing 5% FBS, with replacement of medium every 24 h. The radioactive medium was removed, and 200 were washed with normal medium. The labeled cells were grown until 60% confluent. Tran-[35S]methionine (1,000 Ci/mmol) was purchased from Tran-35S Metabolic labeling of the UMR cells was performed as described by Shah et al. (26). Cells were seeded in 6-well plates and grown until 60% confluent. Tran-[35S]methionine (1,000 Ci/mmol) was added to the cultures and incubated for 24 h. The radioactive medium was removed, and the cells were washed with normal medium. The labeled cells were then incubated in 2 ml/well of normal medium in the presence or absence of 100 nM dexamethasone. At appropriate times, the medium was removed, and 200 µl of RIPA buffer [50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate (DOC), and 0.1% SDS] were added to each well to dissolve cells. The cell extract was then boiled for 20 min to denature it. After centrifugation (15,000 g, 5 min, 4°C), the supernatant was collected. Pansorbin beads (50 µl) were added to the denatured cell extract and incubated at 4°C for 1 h with constant rotation. After centrifugation (15,000 g, 1 min, 4°C), the supernatant was collected and immunoprecipitated by addition of 3 µl of G11α antisera and incubated at 4°C with constant rotation for 1 h. Pansorbin beads (50 µl) were subsequently added to the immune complex and incubated at 4°C overnight with constant rotation. After centrifugation (15,000 g, 1 min, 4°C), the beads were washed three times with 65 µl of wash buffer (50 mM Tris, 150 mM NaCl, and 1% NP-40), resuspended in Laemmli sample buffer, and boiled at 100°C for 5 min. After a final centrifugation (15,000 g, 1 min, 4°C), the supernatant was collected and subjected to SDS-PAGE (13% acrylamide gel). After resolution of the proteins, the gel was stained with Coomassie blue, dried, and exposed to phosphor screen and scanned with Phospholmager (Molecular Dynamics, Sunnyvale, CA). The intensity of the signal was quantified using ImageQuant software.

Northern blot analysis. Total RNA was extracted from UMR cells after dexamethasone treatment using TRizol reagent in accordance with the manufacturer’s protocol (Life Technologies, Burlington, ON). The concentration of RNA was determined by absorbance at 260 nm, and RNA integrity was assessed by gel electrophoresis on 2% agarose gel. For Northern blot analysis, 2.5 µg of denatured total RNA were separated on a 1.2% agarose gel containing 3.7% formaldehyde and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Oakville, ON) by capillary transfer with sodium phosphate (100 mM) transfer buffer. The transferred RNAs were immobilized by ultraviolet cross-linking and prehybridized overnight at 42°C in a solution containing 100 mM sodium phosphate, 1 mM EDTA, 7% SDS, 10 mg/ml BSA, and 0.045 mg/ml RNase-free RNase. The G11α cDNA probe was obtained by cutting the plasmid pCISGo11 (29) with Clal and XhoI to yield a 1,200-bp fragment that represented the full-length G11α cDNA. A probe specific to 18S rRNA was made by PCR amplifying the first-strand cDNA with 18S-specific primer pairs (Ambion, Austin, TX). The amplified product (350 bp) was electrophoresed on agarose gel, excised, and gel purified (Qiagen, Mississauga, ON). These probes were labeled using the random priming method with [α-32P]dCTP and purified using G-50 sizing columns (Roche Molecular Biochemicals, Laval, QC). Radiolabeled probes (17 × 106 counts·min·µg−1·µg−1) were added to the hybridization solution and hybridized overnight at 42°C. The membranes were then washed for 20 min in each of the following solutions: 2× SSC-0.1% SDS-0.5× SSC-0.1% SDS and 0.1× SSC-0.1% SDS. The membranes were exposed to phosphor screen, and the signals were quantified by use of ImageQuant software.

Presentation of data. The results presented were obtained from at least two independent experiments performed on cell cultures between passages 22 and 28. Results of some experiments are expressed as values (means ± SE) of separate experiments. Statistical significance was determined using Student’s t-test.

RESULTS

Characterization of Gα11 and G11α protein expression in UMR 106–01 cells. Our laboratory has previously demonstrated that dexamethasone increased Gα11α protein expression and PLC activity (16). Because the antisera used previously for quantitation recognized both Gα11 and G11α proteins, we wanted to determine which one of the two G protein subtypes was expressed in our cells and upregulated by dexamethasone to increase PLC activity. Using antisera raised against sequences unique to Gα11 and G11α subunits, we found that G11α is the primary PLC-activating G protein expressed in UMR cells and that Gα11 was not detected (Fig. 1A). On the basis of this finding, we used the G11α-specific antibody to reevaluate the effect of dexamethasone on G11α protein expression. Treatment with 100 nM dexamethasone for 3 days increased G11α protein expression fivefold (Fig. 1B), indicating that
Dexamethasone is a potent regulator for G\(_{11}\alpha\) expression in these cells. As shown in Fig. 2A, dexamethasone elevated the level of G\(_{11}\alpha\) in a time-dependent fashion. Significant increase in G\(_{11}\alpha\) protein was observed after 48 h of incubation with dexamethasone and continued to increase after 72 h. No further increases in G\(_{11}\alpha\) protein were seen after longer incubations with dexamethasone (data not shown). Dexamethasone stimulation of G\(_{11}\alpha\) protein expression was dose dependent (Fig. 2B), with maximal stimulation of G\(_{11}\alpha\) protein expression seen with 100 nM dexamethasone treatment, which did not increase further with higher concentrations.

**Effect of dexamethasone on G\(_{11}\alpha\) protein stability.** To investigate whether the elevated steady-state G\(_{11}\alpha\) protein level was due to increased stability of the protein, UMR cells were incubated with 35S-labeled methionine for 24 h, and the loss of radio-labeled G\(_{11}\alpha\) protein with time was monitored in cells that were either untreated or treated with 100 nM dexamethasone. Antiserum CQ2, which recognized both G\(_q\) and G\(_{11}\alpha\), was used to immunoprecipitate G\(_{11}\alpha\) from the UMR cell extracts. The rate of loss of 35S-labeled G\(_{11}\alpha\) protein was slower in cells treated with dexamethasone (Fig. 3). In untreated UMR cells, the half-life (t\(_{1/2}\)) for G\(_{11}\alpha\) protein was estimated to be ~20.3 h, whereas the glucocorticoid-treated cells demonstrated a threefold increase in t\(_{1/2}\) for G\(_{11}\alpha\) to 63 h.

**Effect of dexamethasone on steady-state G\(_{11}\alpha\) mRNA level.** To assess whether changes in G\(_{11}\alpha\) mRNA expression could also contribute to the glucocorticoid-induced increase in steady-state G\(_{11}\alpha\) protein expression, the effect of dexamethasone on G\(_{11}\alpha\) mRNA level was determined by Northern blot analysis. With use of a 32P-labeled mouse G\(_{11}\alpha\) cDNA probe, a single mRNA transcript of ~4 kb was detected in UMR cells, as reported previously in brain (29). Incubation of the cells with 100 nM dexamethasone induced a significant 70% increase in G\(_{11}\alpha\) mRNA expression after 24 h of hormone treatment (Fig. 4A). Longer exposure to glucocorticoids in UMR cells produced no further increase in G\(_{11}\alpha\) mRNA level, but levels remained significantly higher than those of the untreated cells. The increase in G\(_{11}\alpha\) mRNA by glucocorticoids was also found to be dose dependent, with increased levels seen after 24-h treatment with 1 nM dexamethasone and a maximal increase seen with 100 nM dexamethasone (Fig. 4B).

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**Fig. 1.** A: relative levels of G\(_q\) and G\(_{11}\alpha\) protein in UMR 106–01 cells. Brain (B), liver (L), or UMR (U) cell membranes were subjected to SDS-PAGE and then immunoblotted using G\(_q\), G\(_{11}\alpha\), and G\(_q/11\alpha\)-specific antiserum as described in EXPERIMENTAL PROCEDURES. B: effect of dexamethasone on expression of G\(_{11}\alpha\) protein in UMR 106–01 cells. Cells were incubated with 100 nM dexamethasone (Dex) or 0.001% ethanol (EtOH, vehicle) for 72 h. Membranes (40 μg) were subjected to SDS-PAGE and immunoblotted using a G\(_{11}\alpha\)-specific antipeptide antiserum, as described in EXPERIMENTAL PROCEDURES. Bars represent means ± SE of 3 experiments (representative blot shown in inset). Values significantly different from control, *P < 0.05.
The dose dependence of increases in steady-state \( G_{11\alpha} \) mRNA level was more sensitive to low concentrations of dexamethasone (1 nM) compared with its effect on steady-state protein level.

**Mechanism of dexamethasone-induced \( G_{11\alpha} \) mRNA induction.**

The steady-state mRNA level can be regulated by altering the transcription of the gene and/or the stability of the RNA transcript. To determine whether the stimulatory effect of glucocorticoid on \( G_{11\alpha} \) mRNA levels was due to changes in mRNA transcript stability, UMR cells were pretreated with or without 100 nM of dexamethasone for 24 h and then exposed to the transcriptional inhibitor DRB (150 \( \mu \)M) in the absence or presence of the glucocorticoid for 0.5–48 h. The \( G_{11\alpha} \) mRNA transcript remained stable for the first 8 h and then began to decrease; the \( t_{1/2} \) for \( G_{11\alpha} \) mRNA was \( \sim 37.5 \) h, and dexamethasone treatment did not affect the stability of the transcript in these cells (Fig. 5A).

A transcription inhibitor, actinomycin D, was used to investigate whether de novo RNA synthesis was required for the observed increase in steady-state \( G_{11\alpha} \) mRNA levels by dexamethasone. UMR cells were treated for 24 h with dexamethasone (100 nM) in the presence or absence of 3 ng/ml of actinomycin D. Under this condition, actinomycin D effectively inhibited transcription without having toxic effects on the cells. As shown in Fig. 5B, actinomycin D alone had no inhibitory effect on \( G_{11\alpha} \) mRNA expression in control cells, but it completely blocked the dexamethasone-induced stimulation of \( G_{11\alpha} \) mRNA expression.

**Characterization of dexamethasone-mediated response on \( G_{11\alpha} \) mRNA.** As shown in Fig. 4A, there were no significant changes in \( G_{11\alpha} \) mRNA expression by dexamethasone for the first 12 h, but it was increased by 70% after 24 h. In addition, cycloheximide alone decreased control levels of \( G_{11\alpha} \) mRNA expression by \( \sim 40\% \) and completely abolished the dexamethasone-induced stimulation of \( G_{11\alpha} \) mRNA expression (Fig. 6). Together, these results indicate that the glucocorticoid effect on \( G_{11\alpha} \) transcription was a secondary response.

**DISCUSSION**

PTH stimulation of the PTH/PTHrP receptor on osteoblastic cells leads to stimulation of both \( G_{\alpha}\) and \( G_{q/11\alpha} \), activating both the AC and PLC pathways (11). Our previous work in the UMR cell line has demonstrated that glucocorticoids increased PTH activation of both of these pathways, although the effect on the PLC pathway was greater than the increase in AC (16). The ability of glucocorticoids to regulate the AC path-
way has been well documented in many different tissues and cell lines, and it appears to result from increased expression of Gsα as well as of receptors (3, 4, 20, 24). In contrast, the effect of glucocorticoids on the PLC pathway has not been well studied. Our own study in the UMR cells was the first to demonstrate an increase in receptor-stimulated PLC activity and Gq/11α proteins by glucocorticoids (16). This was followed by the demonstration of a similar upregulation of Gq/11α and vasopressin-stimulated PLC in rat pituitary glands after glucocorticoid administration in vivo to rats (18). These studies suggested that glucocorticoid regulation of hormone-stimulated PLC signal transduction may occur in many different tissues, similar to their effects on the AC system, and therefore we continued to explore the molecular mechanism by which the levels of Gq/11α proteins are increased.

In the present report, we demonstrate that the predominant PLC-stimulating G protein expressed in the UMR cell line was G11α. Gqα protein was not detected in our assays; however, we have been able to detect low levels of mRNA encoding Gqα in these cells by RT-PCR, suggesting that there may be very low levels of the Gqα protein that were undetectable in our immunoblotting assays. Clearly, the G11α protein is the major protein of the Gq family expressed in these cells and accounts for all of the protein detected by the Gq/11α antibody.

After incubation with the synthetic glucocorticoid dexamethasone, G11α protein levels increased approximately fivefold within 72 h. This increase in G11α protein was accompanied by a more modest increase (~70%) in G11α mRNA level. This increase was not the result of changes in the stability of G11α mRNA but likely was the result of a stimulation of G11α transcription, because it was not seen in the presence of the transcription inhibitor actinomycin D. The glucocorticoid effect on G11α steady-state mRNA levels appeared to be a secondary response to stimulation of the synthesis of some other regulatory protein, because the increase in G11α mRNA was not seen in the presence of the protein synthesis inhibitor cycloheximide. Furthermore, the time course of dexamethasone-induced increases in G11α mRNA is more consistent with a secondary response. Primary glucocorticoid responses involving binding of agonist to the glucocorticoid recep-

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**Fig. 5.** Mechanism of dexamethasone-induced G11α mRNA induction.

A: effect of dexamethasone on stability of G11α mRNA in UMR 106–01 cells. Cells were incubated with 100 nM dexamethasone (●) or 0.001% ethanol (○) for 24 h, followed by addition of 150 μM DRB for indicated times. B: effect of transcription inhibitor actinomycin D (AD) on dexamethasone (Dex)-mediated response to G11α mRNA. Cells were treated for 24 h with 100 nM dexamethasone or 0.001% ethanol (vehicle) in the absence or presence of 3 ng/ml actinomycin D. Total RNA was extracted at the indicated time points and subjected to Northern blot analysis. RNA (2.5 μg) was loaded and resolved by 2% agarose gel electrophoresis. Transferred RNA blots were hybridized with 32P-labeled rat G11α and 18S cDNA and visualized by autoradiography. Bands were quantified using ImageQuant software as described in EXPERIMENTAL PROCEDURES. Each point represents mean ± SE of 2 individual experiments (representative blot shown in inset). Values significantly different from control, *P < 0.05.

**Fig. 6.** Effect of cycloheximide (CX) on dexamethasone-induced increases in G11α mRNA expression in UMR 106–01 cells. Cells were treated for 24 h with 100 nM dexamethasone or 0.001% ethanol (vehicle) in the absence or presence of 1 μM CX. Total RNA was extracted and subjected to Northern blot analysis. RNA (2.5 μg) was loaded and resolved by 2% agarose gel electrophoresis. Transferred RNA blots were hybridized with 32P-labeled rat G11α and 18S cDNA and visualized by autoradiography. Bands were quantified using ImageQuant software as described in EXPERIMENTAL PROCEDURES. Each point represents mean ± SE of 3 individual experiments (representative blot shown in inset). Values significantly different from control, *P < 0.05.
tor, nuclear translocation, and binding to the glucocorticoid response element have all been shown to occur within 30 min (2). Therefore, the long delay period of 12–24 h before dexamethasone-mediated induction of G11α mRNA is not consistent with a primary response.

The magnitude of the increase in G11α mRNA that we have seen in UMR cells was far less than that previously reported for the effect of dexamethasone on Gα mRNA in GH3 cells, which were increased fivefold after 72 h (3). This suggested that the increase in G11α mRNA may not have been the primary mechanism regulating G11α protein levels in the UMR cells. Indeed, further investigation demonstrated a profound effect of glucocorticoids on the stability of G11α protein, increasing the t1/2 of the protein from 20.3 to 63 h. This is the first report of glucocorticoid-induced changes in G protein stability. Previous studies have reported an increase in the Gα protein stability with changes in t1/2 from 50 to 72 h after triiodothyronine treatment of neonatal rat ventricular myocytes (1). Several studies have also demonstrated that G protein stability can be decreased after prolonged activation of cells by hormones (9, 15, 17, 26, 30, 31) or direct activation of Gα by cholera toxin (3, 14). Together, these reports suggest that the rate of degradation of G proteins is regulated by the cellular hormonal environment and as a consequence may determine the responsiveness of G protein-coupled systems.

The effect of glucocorticoids on protein turnover is tissue specific. In muscle, glucocorticoids have significant proteolytic effects associated with inhibition of protein translation initiation (27), as well as increased proteasome-dependent and calcium-dependent proteolytic pathways (5, 32). In liver, on the other hand, glucocorticoids stimulate gluconeogenesis in part by increasing the production of key hepatic enzymes (8). In the osteoblastic cells used in our study, dexamethasone increased total protein content of the cells by ~30% over 3 days, suggesting a general anabolic effect. This increase in total protein content, as well as the larger increase in specific G protein subunits, may reflect effects of the steroids on cellular proteolytic pathways. There are no reports in the literature of which degradative pathways govern heterotrimeric G proteins in osteoblastic cells and how these are influenced by glucocorticoids. We are currently pursuing studies to determine which proteolytic pathways degrade G proteins in the osteoblastic cells and how these are influenced by glucocorticoids.

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