Modulation of Glucocorticoid Receptor Transcriptional Activation, Phosphorylation, and Growth Inhibition by p27Kip1*

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The cyclin-dependent kinase inhibitor p27Kip1 is frequently inactivated in human cancers. Glucocorticoids, acting through the glucocorticoid receptor (GR), are frequently used to treat certain malignancies and are growth inhibitory, but the relationship between GR activity and p27 status has not been explored. We have therefore examined GR-dependent transcriptional activation, receptor phosphorylation, and glucocorticoid-dependent growth inhibition in p27-deficient (p27−/−) murine embryonic fibroblasts (MEFs). We find that GR transcriptional enhancement as well as receptor phosphorylation at two putative cyclin-dependent kinase sites are elevated in p27−/− MEFs, relative to control cells. This increased GR transcriptional activation appears to be mediated through the GR N terminus, and coexpression of the GR N-terminal coactivator, DRIP150, further enhanced GR-dependent transcriptional activation. Furthermore, p27−/− MEFs are partially resistant to the growth inhibitory effects of glucocorticoids. Thus, p27 appears to be an important element in the GR transcription and growth inhibitory responses.

Glucocorticoids are a class of steroid hormones that govern metabolism and development and are used clinically for the treatment of inflammatory diseases as well as certain types of malignancies. The glucocorticoid signal is conveyed through an intracellular ligand-activated transcription factor, termed the glucocorticoid receptor (GR)1 (1). Transcriptional responses triggered by the hormone-activated GR include both activation and repression depending on the DNA sequences in the target promoters (2).

Glucocorticoids serve as the primary signal for activating the transcriptional regulatory functions of GR; however, receptor activity is also modulated by phosphorylation (3, 4). GR is phosphorylated in the absence of hormone, with additional phosphorylation occurring in conjunction with agonist but not antagonist binding (3, 5, 6). Among the phosphorylation sites that have been identified in the mouse GR N terminus, two sites at which increased phosphorylation is observed on hormone binding, Ser-212 and Ser-220, are consensus cyclin-dependent kinase (Cdk) sites and can be phosphorylated by Cdk in vitro (5, 7). In addition, mutations of particular Cdk genes in reconstituted GR signaling system in yeast yield phenotypes similar to those conferred by mutation of the sites themselves (7–10). These findings strongly suggest that changes in the activity of Cdk render GR differentially responsive to glucocorticoid treatment by affecting receptor phosphorylation.

Cdk proteins are a family composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. Regulation of Cdk activity is accomplished by subunit assembly and through proteins that activate (Cdk activators) or inhibit (Cdk inhibitors or CKI) kinase function. The prototype CKI is p27Kip1 (hereafter referred to as p27), which was first identified as a protein that bound and inhibited cyclin E-Cdk2 activity and has been shown subsequently to regulate cell cycle progression by controlling Cdk activity and activity (11–13). p27 is expressed at high levels in differentiated, noncycling cells and is low or absent in proliferating cells (14). Overexpression of p27 in cultured cells induces a G1 cell cycle arrest, whereas its ablation increases the number of cells entering S-phase and delays withdrawal from the cell cycle when mitogens are removed (15). Mice lacking p27 grow larger than their wild-type littermates, consistent with a role of p27 in cellular proliferation (16, 17). Many anti-mitogenic signals lead to the accumulation of p27, which in turn inhibits Cdk activity, leading to cell cycle arrest. For example, the anti-mitogenic effect of glucocorticoids in fibroblasts and certain osteosarcoma cell lines (e.g. SAOS2) is a result, in part, of an increase in the levels of CKI, including p27 (18–21). Because p27 controls cell division, the dysregulation of p27 has been implicated in oncogenesis (22–24). Indeed, p27 is frequently inactivated in human cancers, which correlates with tumor aggressiveness and a poor prognosis (25–27). Thus, p27 is an important regulator of Cdk activity, which affects cell cycle entry and exit as well as the phosphorylation of many substrates (potentially including GR).

As glucocorticoid-dependent growth inhibition involves increased p27, we wanted to investigate the reciprocal pathway in which p27 affects GR activity. To this end we examined GR transcriptional regulation, receptor phosphorylation, and glucocorticoid-dependent growth inhibition using primary embryonic fibroblasts derived from p27-deficient mice.

EXPERIMENTAL PROCEDURES

Murine Embryo Fibroblast (MEF) Generation and Cell Cultivation—MEFs were generated from 14-day postcoitum mouse embryos of p27−/− heterozygote mating. Embryos were harvested, the heads and internal organs were removed, and carcasses were minced using a scalpel in a Petri dish containing fresh medium. The minced embryos were incubated with trypsin at 37 °C for 15 min, triturated through an 18-gauge needle, and then placed in a 15-cm tissue culture dish containing Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 10 units/ml each of...
penicillin and streptomycin (Cellgro), and 2 mM t-glutamine (Cellgro). Cells were incubated for 2 days at 37 °C with 5% CO₂ and then counted and replated to a density of 1 × 10⁶ cells/10-cm dish. A portion of the primary cells was frozen and stored at −80 °C whereas other portions were split every 3 days at a density of 1 × 10⁶ cells/10-cm dish. Primary cells of fewer than six passages were used in all experiments.

**Genotyping**—To isolate total genomic DNA from mouse embryos, the heads from a single mouse embryo were lysed in 500 μl of lysis buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 0.5% SDS, 25 mM EDTA, 4 μg/ml proteinase K) and incubated at 55 °C for 14–18 h. The DNA was phenol-extracted, ethanol-precipitated, resuspended in 10 mM Tris, pH 8, 0.1 mM EDTA, and used as a template for genotyping via PCR (16).

**Immunoblotting**—Cell extracts from MEFs were separated by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) at 110 V for 80 min in Tris/glycine transfer buffer. The membranes were blocked in 5% bovine serum albumin in Tris-buffered saline (TBS), pH 7.4, overnight at 4 °C. The membranes were incubated in the blocking buffer with primary antibody at room temperature for 2–4 h (1:500 for the p27 monoclonal antibody (Pharmingen; catalog no. 25020); 1:100 dilution of a tissue culture supernatant for the GR mouse monoclonal antibody, BuGR2 (a gift from Keith Yamamoto); and 1:1000 for the Cdk2 rabbit polyclonal antibody (a gift from Michele Pagano). The membranes were washed three times for 10 min in TBS/0.1% Triton X-100 and twice in TBS. Membranes were incubated for 1 h at room temperature with anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase, and washed five times for 10 min in TBS/0.1% Triton X-100; this was followed by TBS, and membranes were developed with an ECL reagent according to the manufacturer's instructions (Amersham Biosciences).

**Immunoprecipitation**—The GR monoclonal antibody, BuGR2, was used for immunoprecipitation. The antibody was prebound to protein A/G agarose beads (Santa Cruz Biototechnology, Inc., Santa Cruz, CA) in phosphate-buffered saline (PBS) at 4 °C for 1.5 h, and washed twice with PBS to remove unbound antibody. BuGR2-coated beads were incubated with cell extracts (1 mg of total protein in lysis buffer per sample) at 4 °C for 3 h on a rocking platform, pelleted by centrifugation, and washed five times in PBS buffer and twice in 50 mM Tris buffer, pH 7.5. The beads were then boiled in 50 μl of SDS sample buffer and analyzed by immunoblotting with an antibody against total mouse GR (BuGR2) or GR phosphorylation state-specific antibodies, mGR-Ser-212-P and mGR-Ser-220-P (6).

**Cdk2 Activity Assay**—Protein extracts were prepared from a subconfluent 16-cm plate of wild-type and p27−/− MEFs. Cells were washed twice with PBS and lysed for 10 min, on ice, in 200 μl of lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.8% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT), supplemented with protease and phosphatase inhibitors: 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml each of aprotinin, pepstatin A, and leupeptin). Cell lysates were clarified by centrifugation (12,000 × g for 15 min at 4 °C) and incubated with 20 μl of protein A/G agarose beads prebound with rabbit anti-Cdk2 antibody for 3 h at 4 °C. The immune complexes were washed three times with lysis buffer and twice with 50 mM Tris, pH 7.5. One-fifth of the beads were removed and boiled in the same volume of 2× SDS sample buffer for 3 min and used to monitor the amount of Cdk2 immunoprecipitated by immunoblotting. The remaining beads were washed in buffer K (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT) three times and resuspended in 30 μl of buffer K. The reaction mixture was incubated for 10 min at 30 °C after adding 8 μl of 1 mg/ml histone H1, 2.5 μl of 1 mM ATP, and 7.5 μCi of [3H]-F1-γ-ATP. The reaction was terminated by the addition of 50 μl of 2× SDS sample buffer and boiled for 3 min. The samples were separated using 4–15% gradient SDS-PAGE, stained, and dried, and autoradiography was performed.

**Transfection of MEFs**—The MEFs were seeded onto 35-mm plates (1 × 10⁶ cells) in DMEM medium for 2 days and refed in phenol red-free DMEM (Cellgro) supplemented with 10% charcoal-stripped fetal bovine serum and 2 mM t-glutamine 2 h before transfection. Cells were transfected using 7 μl of Lipofectamine PLUS (Invitrogen) (4 μl of Lipofectamine PLUS reagent) per 35-mm dish, according to the manufacturer's instructions. The total amount of DNA transfected is held constant in each transfection using the corresponding empty vector. Cells were allowed to recover for 12 h and treated with 100 nM dexamethasone or an ethanol vehicle for 18 h. If no treatment was indicated, cells were harvested 18 h after transfection. Transfected cells were washed twice in PBS and harvested in 1× reporter lysis buffer (Promega) according to the manufacturer's instructions. Luciferase activity was quantified in a reaction mixture containing 25 mM glycyl-glycine, pH 7.8, 10 mM MgSO₄, 1 mM ATP, 0.1 mg/ml bovine serum albumin, and 1 mM DTT, using an LB9507 luminometer (EG&G Berthold) and 1 mM t-luciferin as substrate. The transcriptional activity of GR and Gal4 DNA binding domain fusion proteins were normalized to β-galactosidase activity (an internal control for transfection efficiency) and to protein concentration as determined by the Bradford protein assay (Bio-Rad).

**Flow Cytometry Analysis**—Wild-type and p27−/− cells were cultured with dexamethasone (100 nM) or an ethanol vehicle for 48 h. Cell cycle distribution was assessed by flow cytometry. Cells were trypsinized, pelleted, resuspended in 0.5 ml of 1% fetal calf serum in PBS, and fixed by adding 5 ml of 80% ethanol (at −20 °C) to the solution with continuous vortexing. Fixed cells were pelleted and resuspended in 375 μl of 1% fetal calf serum-PBS. A 4× propidium iodide solution (200 μg of propidium iodide/ml in 38 mM sodium citrate) was added to the reaction mixture and incubated at 37 °C in the presence of 100 μg/ml RNase A. Nuclear-emitted fluorescence was measured under a FACSscan flow cytometer (BD Biosciences), and the percentage of cells in each phase of the cell cycle was determined.

**RESULTS**

**p27 Status Affects GR Transcriptional Activation and Phosphorylation**—To examine the effect of p27 on GR transcriptional activation, we transiently transfected early passage "primary" wild-type and p27−/− MEFs with a GR-responsive reporter plasmid and assessed GR transcriptional activation as a function of dexamethasone concentration. Both wild-type and p27−/− MEFs contained equivalent amounts of endogenous murine GR (Fig. 1A). Relative to the wild-type cells, the p27−/− MEFs displayed an increase of greater than 2-fold in GR transcriptional enhancement after dexamethasone stimulation (Fig. 1B). The maximum GR transcriptional response was achieved at 10−7 M dexamethasone in both wild-type and p27−/− MEFs (Fig. 1B). The level of basal GR-mediated transcriptional activation was not affected by p27 expression. Thus, GR is more efficient at engaging in the interactions necessary for transcriptional activation...
for transcriptional activation in the p27−/− MEFs compared with wild-type cells.

As expected, the total Cdk2 activity is elevated in p27-deficient cells (Fig. 2A). Because our previous results suggested that GR Ser-212 and Ser-220 were substrates for Cdk2 phosphorylation in vitro, we next examined the phosphorylation status of GR at Ser-212 and Ser-220 using GR phosphorylation state-specific antibodies (6). Consistent with our previous studies on human GR, the murine GR displayed a significant basal level of phosphorylation at Ser-212 but not at Ser-220 (Fig. 2B). Phosphorylation of both residues is increased with dexamethasone treatment, particularly at Ser-220. Significantly, a greater dexamethasone-dependent phosphorylation at both Ser-220 and Ser-212 was evident in the p27−/− MEFs compared with control MEFs expressing p27 (see Fig. 2B, compare lanes 2 and 4 in the top and middle panels). This increased GR phosphorylation is not a function of alterations in expression of the receptor between lines, because equal amounts of GR were present as determined by immunoblotting for total GR (Fig. 2B, bottom panel). Furthermore, dexamethasone dependence increased phosphorylation of GR Ser-220 and Ser-212, two putative Cdk sites in p27−/− cells, correlated with increased dexamethasone-dependent transcriptional activation (Fig. 2C).

To further examine the effect of p27 on GR transcriptional activation, we transiently transfected wild-type and p27−/− MEFs with GR derivatives expressing the individual activation functions and assessed GR transcriptional activation. One GR derivative contains AF-1 and the DNA binding domain from amino acids 1–525 (N525) but lacking the ligand-binding/GR AF-2 domains, along with the TAT3-luciferase reporter. C, Gal4-GR AF-1; or D, with Gal4-GR AF-2 and a p5×Gal4-tk-luciferase reporter. Reporter activity assays were performed as described under “Experimental Procedures” except that ligand was omitted in B and C because these derivatives are constitutively active. Results shown represent a single experiment carried out in duplicate, with error bars representing the range of the mean. Each experiment was repeated three times with similar results.
p27 Affects the GR Response

Effects of Glucocorticoids

To determine whether p27 facilitates functional interactions, we transiently transfected wild-type and p27−/− MEFs with the GR AF-1 coactivator, DRIP150 (28). Coexpression of DRIP150 should further activate AF-1, thereby increasing GR transcriptional enhancement, relative to cells not overexpressing DRIP150, and allow us to compare DRIP150 induction of GR activity in the presence and absence of p27. If p27 and the resulting alterations in GR phosphorylation were important for AF-1 interaction with DRIP150, then the absolute level of GR transcriptional activation should be greater in the p27−/− versus the wild-type cells. As shown in Fig. 4, this appears to be the case. In the p27−/− MEFs, the total GR transcriptional activity is greater when DRIP150 is overexpressed than in wild-type MEFs. The fold induction in GR transcriptional activity by DRIP150 overexpression is also slightly elevated, 1.9-fold induction by DRIP150 in p27−/− MEFs versus 1.5-fold induction by DRIP150 in wild-type MEFs (Fig. 4). Thus, the absolute GR transcriptional activity is enhanced by DRIP150 overexpression in p27-deficient cells, suggesting that the loss of p27 expression facilitates the formation of a functional GR-DRIP150 complex, perhaps through alterations in GR and/or DRIP150 phosphorylation.

p27−/− MEFs Are Partially Resistant to Growth Inhibitory Effects of Glucocorticoids—We next examined whether the p27 status affected glucocorticoid-dependent inhibition of cellular proliferation. Previous results from our laboratory have suggested that the induction of p27 by GR is part of the growth inhibitory pathway evoked by glucocorticoids in some cell types. To test the contribution of p27 to GR-dependent growth inhibition, the cell cycle distribution of wild-type and p27−/− MEFs cultured in the presence or absence of dexamethasone for 48 h was assessed by flow cytometry. Consistent with the growth inhibitory role of p27 in the cell cycle, a greater percentage of p27−/− cells are found in S-phase, relative to wild-type cells. With glucocorticoid treatment, the percentage of cells in S-phase decreased by more than 3-fold in the wild-type MEFs but only 1.4-fold in p27−/− MEFs (Fig. 5). These results suggest that the lack of p27 renders cells partially resistant to the anti-proliferative effects of glucocorticoids.

DISCUSSION

GR inhibits cell proliferation in certain cell types by increasing the levels of CKI, such as p21 and p27. Previous results from our laboratory and others have shown that GR-mediated cell cycle arrest involves enhanced expression of p21 and p27. This study suggests that p27 expression also affects GR activity. In the absence of p27, we observed increased Cdk activity, increased hormone-dependent phosphorylation of GR at Ser-212 and Ser-220 (Ser-203 and Ser-211 in the human GR numbering scheme), residues phosphorylated in vitro by Cdk, and modulation of GR hormone-dependent transcriptional activation. The GR activation domains respond to the lack of p27 differentially. AF-1 activity is increased, whereas AF-2 activity appears to be decreased in the absence of p27. Our findings support a model whereby endogenous p27 normally restricts transcriptional activity of GR-AF-1 while promoting AF-2 activity such that the loss of p27 would enhance GR activation at promoters regulated by AF-1 and reduce GR response at AF-2-dependent promoters.

A well studied function of GR is the induction of apoptosis, which appears to require DNA binding-dependent gene activation by the receptor (29). Interestingly, thymocytes from transgenic mice overexpressing GR show increased GR activity and initiate glucocorticoid-induced apoptosis at a hormone concentration that is 10-fold lower than in thymocytes from wild-type mice (30). Consequently, the strength of GR transcriptional activation can determine the degree of activation-dependent events such as thymocyte apoptosis. As GR activation appears elevated in p27−/− MEFs relative to wild-type cells, we would predict that thymocytes from p27−/− mice would be more sensitive to glucocorticoid-induced apoptosis, provided that the...
presumptive pro-apoptotic genes induced by GR are largely AF-1-dependent. However, it has been previously reported that thymocyte apoptosis induced by GR is not affected by p27 when assayed at a superphysiological hormone concentration (1 μM dexamethasone) (16). In light of the findings that glucocorticoid-dependent apoptosis correlates with GR activation potential and that p27 status serves to modulate the transcriptional activity of the receptor, especially at low physiological levels of hormone, it will be interesting to re-evaluate GR-induced apoptosis in p27−/− and control thymocytes.

Lack of p27 also affects the ability of the receptor to inhibit cell proliferation and renders cells partially resistant to the growth inhibitory effect of glucocorticoids. Recent reports suggest that GR activity is also modulated by the CKI p16INK (31). In an elegant set of experiments, MEFs in which the p16 gene has been deleted exhibited increased GR activity and reduced transcriptional activity (135). Such responses have profound effects on GR transcriptional and anti-mitogenic responses.

The effect of p27 on GR transcriptional enhancement, receptor phosphorylation, and susceptibility of cells to the anti-proliferative effects of dexamethasone most likely result from changes in Cdk activity. These changes may directly affect GR phosphorylation as well as phosphorylation of other substrates that modulate GR transcriptional responses. This observation might have important implications for glucocorticoid treatment of certain malignancies, which may exhibit different sensitivity to the anti-proliferative effects of glucocorticoids, depending on whether or not the cancer cells express p27 (32).

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