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Glucocorticoid Receptor-Mediated Cell Cycle Arrest Is Achieved through Distinct Cell-Specific Transcriptional Regulatory Mechanisms

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Glucocorticoids inhibit proliferation of many cell types, but the events leading from the activated glucocorticoid receptor (GR) to growth arrest are not understood. Ectopic expression and activation of GR in human osteosarcoma cell lines U2OS and SAOS2, which lack endogenous receptors, result in a G_1 cell cycle arrest. GR activation in U2OS cells represses expression of the cyclin-dependent kinases (CDKs) CDK4 and CDK6 as well as their regulatory partner, cyclin D3, leading to hypophosphorylation of the retinoblastoma protein (Rb). We also demonstrate a ligand-dependent reduction in the expression of E2F-1 and c-Myc, transcription factors involved in the G_1-to-S-phase transition. Mitogen-activated protein kinase, CDK2, cyclin E, and the CDK inhibitors (CDIs) p27 and p21 are unaffected by receptor activation in U2OS cells. The receptor's N-terminal transcriptional activation domain is not required for growth arrest in U2OS cells. In Rb-deficient SAOS2 cells, however, the expression of p27 and p21 is induced upon receptor activation. Remarkably, in SAOS2 cells that express a GR deletion derivative lacking the N-terminal transcriptional activation domain, induction of CDI expression is abolished and the cells fail to undergo ligand-dependent cell cycle arrest. Similarly, murine S49 lymphoma cells, which, like SAOS2 cells, lack Rb, require the N-terminal activation domain for growth arrest and induce CDI expression upon GR activation. These cell-type-specific differences in receptor domains and cellular targets linking GR activation to cell cycle machinery suggest two distinct regulatory mechanisms of GR-mediated cell cycle arrest: one involving transcriptional repression of G_1 cyclins and CDKs and the other involving enhanced transcription of CDIs by the activated receptor.

Glucocorticoids are important regulatory molecules that govern metabolism and development (9, 72). They have potent antiproliferative effects in many cell types. Glucocorticoids can induce a G_1 cell cycle arrest and programmed cell death of immature thymocytes, several leukemic cell lines, and mature peripheral T lymphocytes (1, 63, 74). They also inhibit the proliferation of mammary epithelial cells (4, 22, 69), fibroblasts (18), and hepatoma cells (11, 52) and induce differentiation of osteoblasts (46, 54). Although these diverse effects of glucocorticoid hormones have been well documented, the mechanism by which these effects are induced remains unclear.

The glucocorticoid signal is conveyed through an intracellular ligand-activated transcriptional regulatory protein termed the glucocorticoid receptor (GR). A member of the steroid receptor superfamily, GR has a characteristic modular structure, with individual domains performing distinct functions. The N-terminal segment of GR contains a transcriptional activation domain. The central zinc-finger region includes the DNA binding domain that not only determines target gene specificity but also mediates interaction between the receptor and the transcription factor AP-1 (14, 53). The hormone binding region, nuclear localization signal, and an additional transcriptional regulatory function are located at the C-terminal part of the molecule. In conjunction with hormone binding, the receptor binds to specific glucocorticoid response elements (GREs) in target genes and modulates transcription from nearby promoters (64). A simple GRE consists of an imperfect palindrome with two hexamer half-sites separated by 3 bp (6, 30, 72). Receptor binding to such elements leads to transcriptional activation (69). Depending on the structure of the GRE and the composition of transcriptional regulatory proteins bound at the site, GR can also repress transcription. For example, GR can inhibit AP-1-mediated transcriptional activation through a protein-protein interaction (14, 37, 75, 78).

Cell cycle progression is regulated through the coordinated expression, posttranslational modification, and degradation of cyclins and their catalytic partners, cyclin-dependent kinases (CDKs). Cyclins, expressed at particular stages of the cell cycle, associate with specific CDKs to form active kinase complexes which phosphorylate multiple cellular proteins and promote cell cycle progression. Cyclin-CDK complexes are negatively regulated by a family of CDK-inhibitory proteins (CDIs) which bind to cyclin-CDK pairs and inhibit their catalytic activity, ultimately impeding cell cycle progression (33). Targets of cyclin-CDK action include a family of pocket proteins, p107, p130, and the retinoblastoma gene product (Rb), all of which are inactivated by CDK phosphorylation during the G_1 phase of the cell cycle (41, 56). The down-regulation of specific G_1 kinases or inhibition of their activity by CDIs results in Rb hypophosphorylation (55, 57). This form of Rb sequesters the transcriptional regulatory protein E2F-1 in an inactive complex (47, 71), inhibiting expression of E2F-responsive genes necessary for DNA replication (e.g., thymidine kinase [44], DNA polymerase alpha [13, 40, 50], and dihydrofolate reductase [3, 25, 66]), which ultimately prevents entry into the S phase. Conversely, when Rb is phosphorylated by D- and E-type cyclin-CDK complexes, E2F-1 dissociates from Rb to activate transcription, allowing the G_1-to-S-phase transition (70).

The mechanism of cell growth arrest by GR remains unclear. However, since the receptor can both enhance and repress

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gene expression, two models can be proposed. One holds that the hormone-activated receptor induces antimitogenic factors, such as CDIs, which cause cell cycle arrest. An alternative hypothesis suggests that GR evokes growth arrest not as a transcriptional activator, but as a transcriptional repressor, interfering with the expression of mitogenic factors, such as cyclins and CDKs.

Previous attempts to define the receptor domains, target genes, and transcriptional regulatory mechanisms that underlie GR-mediated cell cycle arrest have utilized glucocorticoid-sensitive lymphocytes that undergo ligand-dependent growth arrest and apoptosis. These studies, however, provided a rather controversial picture of the mechanism of receptor-mediated cell cycle arrest. For example, in the human leukemic cell line CEM, the zinc-finger region appears sufficient for growth arrest and apoptosis, while the N- and C-terminal transcriptional activation domains are dispensable (42, 62). In contrast, the N-terminal transcriptional activation domain of the receptor is required for growth arrest and apoptosis of mouse S49 lymphoma cells (7, 15, 36, 58, 74). Furthermore, substitution of this domain with sequences from the transcriptional activation domains of VP16 or E1A results in growth arrest and apoptosis (7, 15). These results implicate transcriptional activation as the primary mode of receptor-induced growth arrest in mouse S49 cells. In contrast to this study, transcriptional repression was proposed to be the mechanism of ligand-induced growth arrest in human Jurkat T cells (23).

These discrepancies in the receptor domains and mechanisms of transcriptional regulation involved in growth control have been attributed to inherent interspecies differences in receptor signaling, potential discrepancies in receptor expression levels, or subtle differences in the receptor derivatives used in various experiments. However, none of these explanations is entirely satisfying, nor have these possibilities been examined experimentally. It appeared more likely that the differences in the GR regions governing cell cycle arrest reflect alternative modes of receptor transcriptional regulation—activation versus repression—that function to inhibit cell proliferation depending on the cell context.

To further elucidate the mechanism of GR-mediated control of cell proliferation and to investigate potential cell-type-specific differences in receptor signaling, we have established a cultured cell system with the human osteosarcoma cell lines U2OS and SAOS2. These cells were chosen for two reasons. First, they lack endogenous GR, allowing ectopic expression of wild-type (wt) and mutant forms of the receptor for the study in detail of the specific receptor domains, as well as the type of receptor-mediated transcriptional regulation involved in cell growth control. Second, the compositions of cell-cycle-regulatory proteins differ between the two lines, thus providing distinct cellular environments in which to examine the effects of receptor activation on cell proliferation. U2OS cells express the retinoblastoma gene product Rb, the tumor suppressor protein p53, and three types of D cyclins (D1, D2, and D3) (17). In contrast, SAOS2 cells are p53 and Rb deficient and express only cyclin D3 (17). GR may utilize different transcriptional regulatory mechanisms and target genes to affect cell proliferation depending on cell-type-specific background.

Here we demonstrate that a single transcription factor, the GR, can induce cell cycle arrest through distinct transcriptional regulatory mechanisms depending on the cell type. These findings have broad implications for cell-type-specific regulation of gene expression and cell growth control. In addition, our results provide a molecular basis for the antiproliferative effects of GR activation in different cells, which may have important implications for glucocorticoid use in clinical oncology.

MATERIALS AND METHODS

Cell lines and treatments. Human osteosarcoma cell lines U2OS and SAOS2 were obtained from the American Type Culture Collection (ATCC HTB 96 and 85, respectively) and maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, Utah)–50 U each of penicillin and streptomycin (Gibco BRL) per ml–2 mM L-glutamine (Gibco BRL). Mouse lymphoma S49.1 cells and glucocorticoid-resistant mutant S49.1TB.4 DEX R.63 cells were obtained from the American Type Culture Collection (ATCC TIB 28 and 33, respectively) and cultured in DMEM, supplemented with 10% FBS–50 U each of penicillin and streptomycin per ml–2 mM L-glutamine. Human Jurkat cells were provided by J. Schlessinger and cultured in RPMI medium 1640 (Gibco BRL), supplemented with 10% FBS–50 U each of penicillin and streptomycin per ml–2 mM L-glutamine.

To generate cell lines ectopically expressing wt GR or GR derivatives, 60% confluent U2OS and SAOS2 cells were transfected with pCMV-GR, pCMV-Δ4C1, or pCMV-NLxC expression vector (15 to 20 μg of DNA per 100-mm dish) by the calcium phosphate procedure (2). Stable transformants were selected by culturing transfected cells in the presence of 800 μg of geneticin (G418; Gibco BRL) per ml for 2 months and were further maintained in 10% FBS–DMEM supplemented with 400 μg of G418 per ml. To examine the effects of GR activation on cell proliferation, cell cycle distribution, protein expression, and steady-state mRNA levels, receptor-expressing and control cells were cultured in 10% FBS–DMEM supplemented with 100 nM dexamethasone (DEX; Sigma, St. Louis, Mo.; dissolved in 100% ethanol) or an equal volume of 100% ethanol for the indicated number of days. To arrest de novo protein synthesis, cells were cultured in the presence of 10 μg of cycloheximide per ml for 2.5 h.

Plasmids and cDNAs. The full-length rat GR was subcloned into the *Bam*HI site of the pCMV-Neo^r expression vector in both sense and antisense orientation. The GR deletion mutant, lacking the N-terminal transcriptional regulatory domain between amino acids 70 and 300 (Δ4C1), and a chimeric NLxC derivative with the GR DNA binding domain substituted with that of a LexA bacterial DNA-binding protein were subcloned into the *Bam*HI site of a pCMV-Neo^r expression vector.

For the labeling reaction in Northern blot analysis, full-length E2F-1 and CIP1 (p21) cDNAs were excised from Bluescript (pBS) *Bam*HI and *Xho*I sites, respectively. A c-*myc* fragment of approximately 1.5 kb containing exons 2 and 3 was released by *Hin*dIII-*Eco*RI digestion. A 677-bp *Pvu*II fragment of CDK4 cDNA was excised from pRSV-CDK4 plasmid.

Cell proliferation assays and flow cytometry analysis. U2OS and SAOS2 cell lines ectopically expressing wt GR or receptor deletion and substitution derivatives were seeded into six-well plates (15,000 and 20,000 cells per well, respectively) on day 0 and cultured in the presence of 100 nM DEX or the ethanol vehicle (see "Cell lines and treatments" above). On indicated days, cells were trypsinized, resuspended in DMEM, stained by the trypan blue exclusion method, and counted with a hemocytometer.

Cell cycle distribution was assessed by flow cytometry analysis. On indicated days, 1.0×10^6 cells were trypsinized, pelleted, resuspended in 0.5 ml of 1% FBS in phosphate-buffered saline (PBS), and fixed by adding 5 ml of ethanol (−20°C; 80%) dropwise with continuous vortexing. After overnight incubation at 4°C, cells were pelleted and resuspended in 0.75 ml of 1% FBS–PBS. The DNA was stained by adding 0.25 ml of 4× propidium iodide solution (200 μg of propidium iodide per ml in 38 mM sodium citrate) followed by a 2-h incubation at 37°C in the presence of 100 μg of RNase A per ml. Nuclear emitted fluorescence was measured with a FACScan flow cytometer (Becton Dickinson), and the percentage of cells in each phase of the cell cycle was determined with the "Modfit" computer program.

Immunoprecipitations and Western blotting. To immunoprecipitate wt GR, Δ4C1, and NLxC receptor derivatives from U2OS or SAOS2 stable transfectants, cells were cultured in 100-mm dishes, washed twice with PBS, harvested in PBS, and lysed in 0.5 ml of Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris [pH 7.5], with protease inhibitor 1 mM phenylmethylsulfonyl fluoride [Sigma], and 1 μg each of aprotinin, pepstatin A, and leupeptin [Boehringer Mannheim Biochemicals, Indianapolis, Ind.] per ml) for 20 min at 4°C. Cell lysates were clarified by centrifugation (10,000 × *g* for 10 min at 4°C) and incubated with BuGR2 antibodies (19) for 1 h on ice followed by incubation with 25 μl of protein A/G agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) for 1 h at 4°C, nutating. The beads were washed four times in NP-40 lysis buffer and once in the same buffer without detergent and boiled for 2 min in 30 μl of 2× sodium dodecyl sulfate (SDS) sample buffer. Prior to immunoprecipitation, aliquots of the lysates were directly boiled in an equal volume of 2× SDS sample buffer to make whole-cell extracts (WCE).

To analyze changes in protein expression, U2OS and SAOS2 cells were treated for 3 days as described above, harvested in PBS, and lysed in a small volume (100 to 150 μl) of NP-40 lysis buffer for 20 min on ice. S49, mutant S49, and Jurkat cells were treated with 100 nM DEX overnight where indicated, harvested, washed once with PBS, and lysed in 50 μl of NP-40 lysis buffer for 15 min on ice. Lysates were centrifuged as described above, total protein concentration was

adjusted with the lysis buffer, and samples were boiled in an equal volume of 2× SDS sample buffer. For Western blotting, protein extracts were separated by SDS–9, -10, -12.5, or -15% polyacrylamide gel electrophoresis (PAGE); transferred to Immobilon paper (Millipore Corp., Burlington, Mass.); and probed with mouse monoclonal antibodies against GR (BuGR2), E2F-1, cyclin E, cyclin D3 (Santa Cruz Biotechnology, Inc.), p27/KIP1, p21/CIP1, CDK2 (Transduction Laboratories, Lexington, Ky.), and p16 (PharMingen, San Diego, Calif.) or rabbit polyclonal antiserum against Rb, CDK6, mitogen-activated protein kinase (MAP-K) (Santa Cruz Biotechnology, Inc.), and CDK4 (Upstate Biotechnology Inc., Lake Placid, N.Y.). The blots were developed with either alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Hercules, Calif.) followed by the addition of the BCIP (5-bromo-4-chloro-3-indolylphosphate)-Nitro Blue Tetrazolium phosphatase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) or horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse antibodies and the enhanced chemiluminescence (ECL) substrate as per the manufacturer's instructions (Amersham International plc, Little Chalfont, United Kingdom). Stripping and reprobing of the ECL-probed membranes were performed exactly as described in the manufacturer's instructions.

Northern blotting. Cells were cultured in 150-mm dishes for indicated periods of time with appropriate treatments (see figure legends), the media were aspirated, and cells were lysed directly on the dishes by adding 4.5 ml of Ultraspec RNA reagent (BIOTEXC Laboratories, Inc., Houston, Tex.) per dish. Total RNA was isolated from cell homogenates as per the manufacturer's instructions, denatured at 65°C for 15 min, and separated on a 1.2% agarose–6.2% formaldehyde denaturing gel (7 to 10 μg of RNA per lane). Equivalent loading was verified by ethidium bromide staining of rRNA. RNA was transferred to a 0.45-μm-pore-size nitrocellulose membrane (BA85; Schleicher and Schuell, Keene, N.H.) as described elsewhere (51), and the blot was hybridized to cDNA probes for indicated mRNA species. cDNA fragments coding for c-Myc and CDK4 were labeled with [α-^{32}P]dCTP with the nick translation system (Gibco BRL); the probes for E2F-1 and p21/CIP1 were labeled with the RediPrime random priming labeling kit (Amersham) as per the manufacturer's instructions. Blots were washed, dried, and exposed to Kodak BioMax or X-Omat films for 1 to 5 days at −80°C for autoradiography.

RESULTS

Ectopic expression and activation of the GR in U2OS and SAOS2 human osteosarcoma cell lines lead to cell cycle arrest. To generate stable cell lines expressing the GR, U2OS and SAOS2 cells, which lack endogenous GR, were transfected with the rat GR cDNA cloned in either sense (+) or antisense (−) orientation into a pCMV expression vector carrying a neomycin resistance (Neo^r) gene. Multiple neomycin-resistant clones were generated from both cell types, and receptor expression was verified by immunoblotting with the GR-specific monoclonal antibody BuGR2. The full-length receptor was detected in whole-cell lysates and immunoprecipitates from multiple U2OS-GR(+) and SAOS2-GR(+) clones but not from control U2OS-GR(−) and SAOS2-GR(−) cell lines (Fig. 1A and 2A). As determined by immunoblotting, the total number of receptors in either cell line was similar to that expressed in a rat hepatoma cell line (approximately 50,000 receptors per cell [65]) and thus reflects a physiological level of receptor expression (not shown).

We next examined whether GR activation by the glucocorticoid DEX affected the kinetics of cell growth. We performed proliferation assays on U2OS-GR(+) and SAOS2-GR(+) cells and compared cell growth kinetics to those of control receptor-negative clones cultured in the presence or absence of a saturating concentration (10^{-7} M) of DEX. Cell viability was determined by the trypan blue exclusion method. Addition of ligand caused a dramatic suppression of cell growth in both U2OS and SAOS2 cell lines expressing the receptor, whereas receptor-negative control cells were insensitive to DEX treatment (Fig. 1B and 2B). In both cell types, we observed ligand-dependent inhibition of cell proliferation in multiple clones expressing the receptor. In addition to inhibiting cell proliferation, ligand treatment induces changes in cell morphology. For example, U2OS-GR(+) cells extend long fibers and adopt a fibroblast-like appearance after 24 h of addition of DEX to the medium (Fig. 1C). Ligand-treated SAOS2-GR(+) cells adopt a fried-egg or tent-shaped morphology and are much larger than untreated cells (Fig. 2C). Hormone treatment also results in a reduction of viability in U2OS-GR(+) and SAOS2-GR(+) cells, as evidenced by a growing proportion of trypan blue-staining cells. Preliminary studies of nuclear morphology by DNA staining with Hoechst dye 33422 and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assays suggest that activated GR induces apoptosis in U2OS-GR(+) but not SAOS2-GR(+) cells (not shown). No morphological alterations were observed in receptor-expressing cells in the absence of DEX or in control receptor-negative U2OS and SAOS2 cells in the presence of DEX.

We next asked whether the observed ligand-dependent inhibition of cell proliferation in GR-expressing U2OS and SAOS2 cells reflects a stage-specific arrest of the cell cycle. Cell cycle distribution of U2OS-GR(+) and SAOS2-GR(+) clones cultured in the presence or absence of ligand was assessed by flow cytometry. Tables 1 and 2 demonstrate that treatment of receptor-expressing clones of both cell types with 10^{-7} M DEX results in accumulation of cells in the G_1 phase of the cell cycle, with a simultaneous reduction in the fraction of cells in the S phase. U2OS-GR(+) cells start to accumulate in G_1 within 24 h of ligand treatment. The percentage of cells in G_1 continues to increase over the next 48 h (compare 57% of cells in G_1 in the absence of ligand to 79, 80, and 82% in the presence of DEX after 24, 48, and 72 h, respectively, of continuous hormone treatment [Table 1]). The proportion of cells in S phase progressively decreases from 26% in the absence of DEX to 5% after 72 h of ligand treatment, whereas the percentage of cells undergoing mitosis declines from 17 to 13%. Presumably, this time course reflects the fact that an asynchronous population of cells is initially exposed to the hormone and a majority of cells need to complete a cycle before they can be arrested in G_1. A similar accumulation of cells in the G_1 phase of the cell cycle is evident in SAOS2-GR(+) cells after ligand treatment (Table 2). Flow cytometry analysis of SAOS2-GR(+) cells cultured in the presence of DEX shows that the proportion of cells in G_1 increases from 47 to 72%, whereas subpopulations of cells in the S phase and G_2/M phase are reduced from 25 to 9% and from 28 to 19%, respectively. We conclude that activation of the ectopically expressed GR by ligand induces a G_1 cell cycle arrest in both U2OS and SAOS2 cells.

Cellular targets of activated GR in U2OS and SAOS2 cells. A number of mechanisms may account for the G_1 growth arrest in ligand-treated U2OS-GR(+) and SAOS2-GR(+) cells. The receptor may repress the level or activity of growth-promoting factors involved in the G_1-to-S-phase transition, such as the transcriptional regulatory proteins E2F-1 and c-Myc or the cell-cycle-regulatory proteins, the CDKs and cyclins. Alternatively, the receptor may stimulate the production of growth-inhibiting factors, such as the CDIs p21 and p27, which facilitate G_1 arrest. To determine which mechanism(s) may be operating to induce cell cycle arrest in either cell line, we examined the expression of these putative GR targets at the protein and mRNA level. GR-expressing U2OS and SAOS2 cells, as well as receptor-negative control cells, were cultured for 3 days in the absence or presence of 10^{-7} M DEX. WCE were prepared (see Materials and Methods) and subjected to immunoblot analysis with antibodies against a variety of proteins known to regulate cell cycle progression. Ligand treatment of GR-expressing U2OS cells induced the retinoblastoma gene product Rb to undergo a significant mobility shift from its inactive, highly phosphorylated form to its growth-suppressing, hypophosphorylated form (Fig. 3A). In addition, the total amount of the transcription factor E2F-1, an Rb

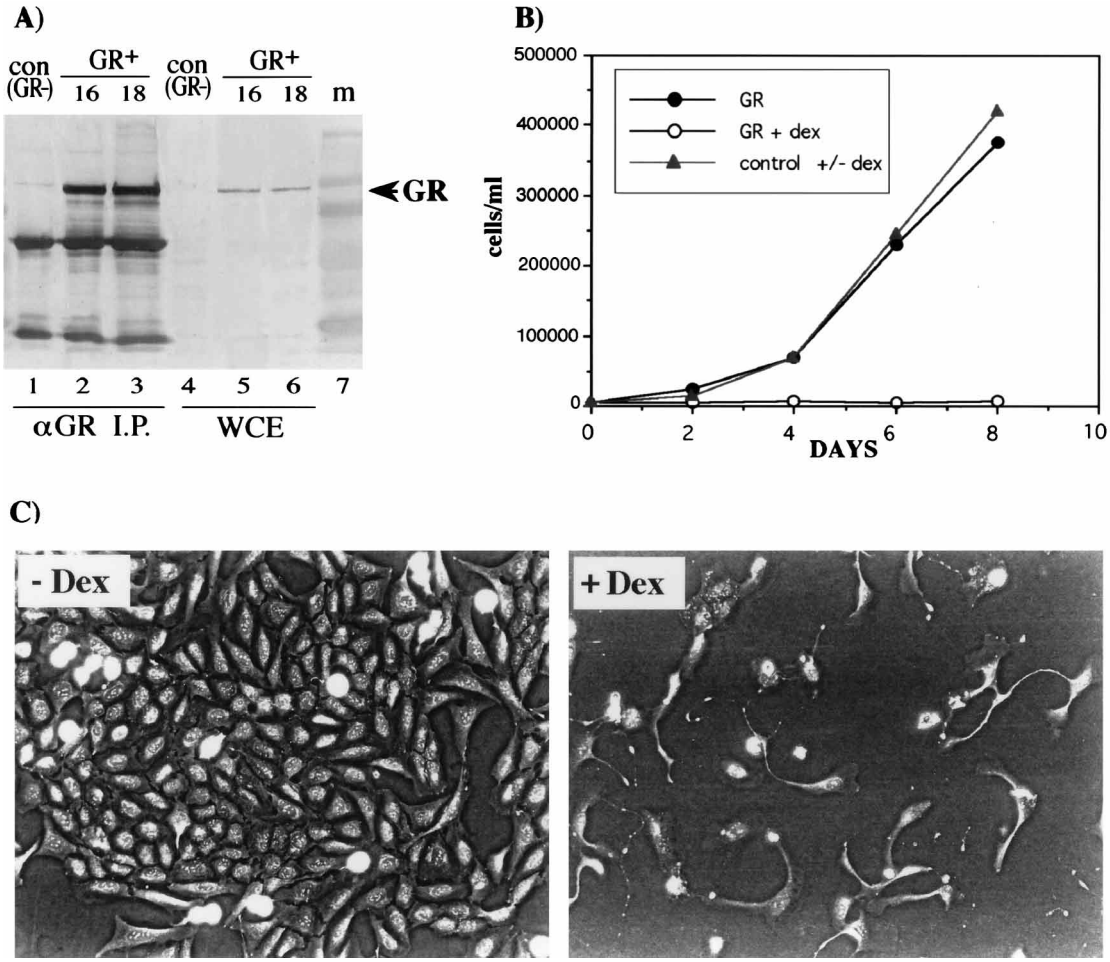

FIG. 1. Effects of GR activation in the human osteosarcoma cell line U2OS. (A) Ectopic expression of GR in U2OS cells. U2OS cells were transfected with rat GR cDNA subcloned into a pCMV-Neo^r expression vector in a sense (+) or antisense (−) orientation, and multiple G418-resistant clones were selected. Stable transformants were tested for GR expression by Western blotting. Cell lysates were prepared from a control clone (GR−, lanes 1 and 4) and GR+ clones 16 and 18 (lanes 2, 3, 5, and 6). A 50-μl aliquot of each sample was saved, and the rest was immunoprecipitated with GR-specific monoclonal antibodies (BuGR2). Both WCE (lanes 4 to 6) and immunoprecipitated proteins (I.P., lanes 1 to 3) were separated on SDS–10% PAGE, transferred to Immobilon paper, probed with BuGR2 antibody, and developed with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody and BCIP-Nitro Blue Tetrazolium phosphatase substrate. Lane 7, molecular mass markers. (B) Cell growth kinetics of U2OS stable lines. U2OS-GR(+) stable transformants or control GR(−) cells (filled triangles) were seeded on day 0 into six-well plates and cultured in the absence (filled circles) or presence (empty circles) of 100 nM DEX. Total numbers of viable cells were determined on the indicated days by the trypan blue exclusion method. The graph represents one of at least five independent experiments. (C) Morphology of U2OS-GR(+) cells. A U2OS cell line stably expressing GR was cultured in the absence (− Dex) or presence (+ Dex) of 100 nM DEX, and phase-contrast photomicrographs were taken on day 3 with Kodak T-MAX ASA400 film.

target, decreased dramatically with hormone treatment (Fig. 3B). We also found that levels of the Rb kinases CDK4 and, to a lesser degree, CDK6, as well as cyclin D3, were decreased in GR-expressing cells treated with ligand but not in untreated GR(+) cells or in control GR(−) cells (Fig. 3C, D, and F). The expression of cyclin E and the CDIs p21 and p27 was unaffected by ligand treatment in U2OS-GR(+) cells (Fig. 3E and G). Figures 3H and I demonstrate that the levels of CDK2 and MAP-K/ERK-1 were also not affected by ligand treatment.

The results of the immunoblot analyses of U2OS-GR(+) cells are confirmed by Northern hybridization of total RNA extracted from receptor-expressing and control cells cultured in the presence or absence of DEX for 24 h. As seen at the protein level, we observed a corresponding reduction in the steady-state mRNA coding for CDK4 and E2F-1 in ligand-treated U2OS-GR(+) cells (Fig. 4B and C). Interestingly, the mRNA level of c-*myc* was dramatically repressed in U2OS-GR(+) cells cultured in the presence of ligand (Fig. 4A). In contrast, the expression of p21 mRNA was unchanged by ligand treatment (Fig. 4D). Together, these results suggest that the observed DEX-dependent growth inhibition in U2OS-GR(+) cells correlates with the receptor's ability to repress the level and/or activity of growth-promoting factors, including CDK4, E2F-1, and c-*myc*.

Surprisingly, in SAOS2 cells, the targets of the activated receptor differ. In SAOS2-GR(+) cells, the expression of E2F-1 is unaffected by ligand treatment at the protein level (Fig. 3B) and is slightly elevated at the mRNA level (Fig. 4C). In addition, the levels of the G_1 kinases CDK4 (Fig. 3C and 4B) and CDK6 (Fig. 3D) were only minimally decreased in ligand-treated cells. As was observed in U2OS-GR(+) cells, the steady-state mRNA level of c-*myc* is repressed in SAOS2-GR(+) cells exposed to DEX (Fig. 4A). Remarkably, the expression of the CDIs p21 and p27 increases severalfold at both the protein and the mRNA levels upon ligand treatment in SAOS2-GR(+) cells (Fig. 3E and 4D). However, not all CDIs

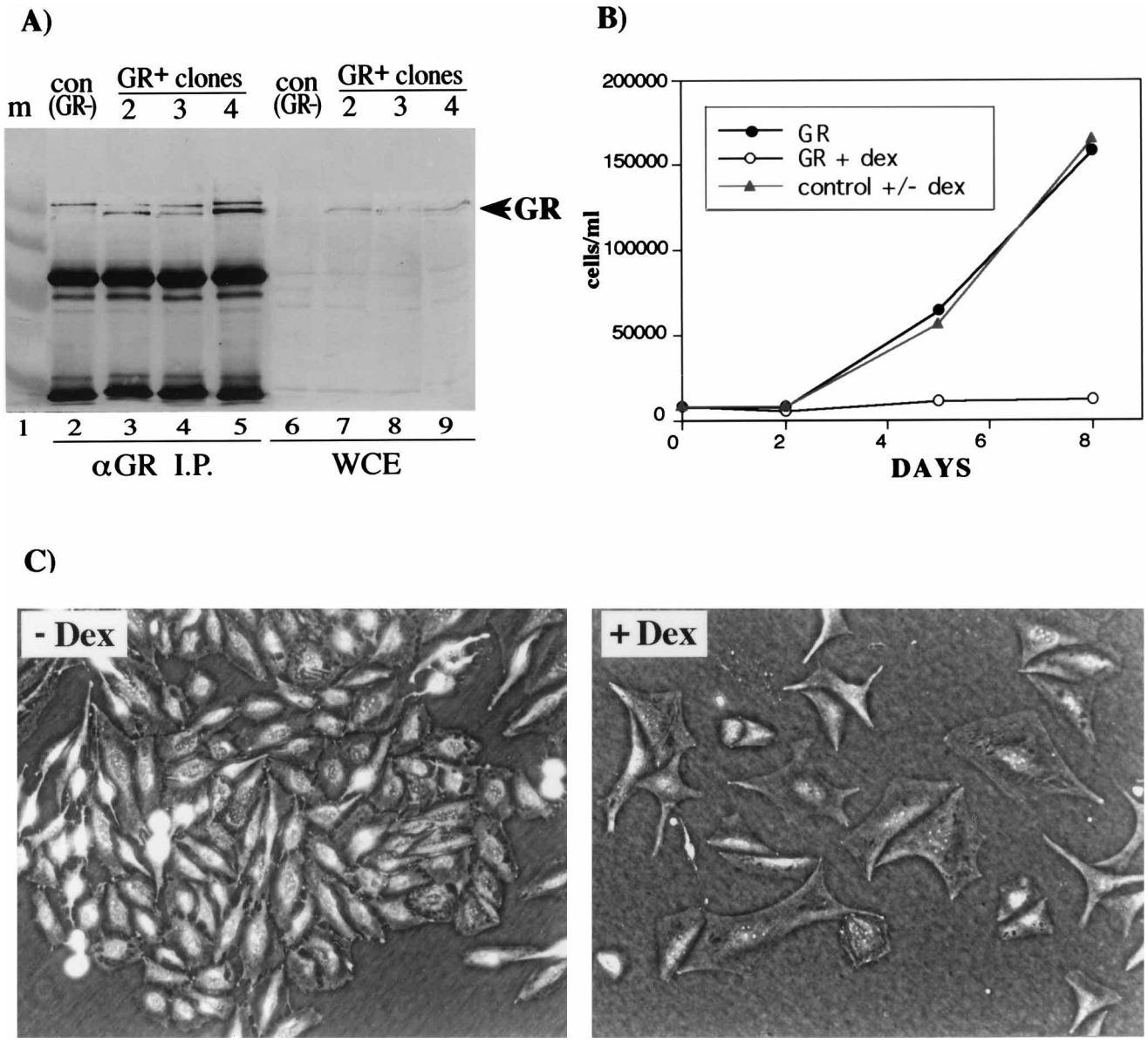

FIG. 2. Effects of GR activation in the Rb-deficient human osteosarcoma cell line SAOS2. (A) Ectopic expression of GR in SAOS2 cells. SAOS2 cells were transfected with rat GR cDNA subcloned into a pCMV-Neo^r expression vector in a sense (+) or antisense (−) orientation, and multiple G418-resistant clones were selected. Cell lysates from a control clone (GR−, lanes 2 and 6) and GR+ clones 2, 3, and 4 (lanes 3 to 5 and 7 to 9) were tested for GR expression as WCE (lanes 6 to 9) or after immunoprecipitation (lanes 2 to 5) as described for Fig. 1A. Lane 1, molecular mass markers. (B) Cell growth kinetics of SAOS2 stable lines. SAOS2-GR(+) stable transformants or control GR(−) cells (filled triangles) were seeded on day 0 into six-well plates and cultured in the absence (filled circles) or presence (empty circles) of 100 nM DEX. Total numbers of viable cells were determined on the indicated days by the trypan blue exclusion method. The graph represents one of at least three independent experiments. (C) Morphological changes in SAOS2-GR(+) cells. An SAOS2 cell line stably expressing GR was cultured in the absence (− Dex) or presence (+ Dex) of 100 nM DEX, and phase-contrast photomicrographs were taken on day 7 with Kodak T-MAX ASA400 film.

are induced by receptor activation. For example, expression of the CDI p16 is slightly repressed in ligand-treated SAOS2-GR(+) cells (Fig. 3E). This contrasts sharply with U2OS-GR(+) cells, in which the expression of p21 and p27 was unaffected by hormone stimulation. These findings suggest that cell cycle arrest in SAOS2-GR(+) cells may be mediated by the transcriptional activating function of the receptor, which induces production of the growth-inhibitory factors p21 and p27.

Our findings thus highlight important differences between U2OS and SAOS2 cells in the events leading to GR-dependent cell cycle arrest. The Rb protein appears to be a central target of the activated receptor in U2OS-GR(+) cells. As a result of GR-mediated repression of the Rb kinases CDK4 and CDK6 as well as their regulatory partner, cyclin D3, Rb fails to undergo phosphorylation, which normally precedes the G_1-to-S-phase transition. Instead, the active hypophosphorylated form of Rb sequesters E2F-1 in a transcriptionally inactive complex, thus preventing the expression of E2F-1-responsive genes, including c-*myc* and E2F-1 itself. The consequence of this cascade of events is the hormone-dependent accumulation of U2OS-GR(+) cells in the G_1 phase of the cell cycle. In contrast, receptor activation in the Rb-negative SAOS2-GR(+) cells appears to increase dramatically the level of the CDIs p21

TABLE 1. Changes in cell cycle distribution of U2OS-GR(+) cells as a result of DEX treatment[a]

Treatment or day	% Fluorescence at cell cycle phase:		
	G_0/G_1	S	G_2/M
No hormone	57	26	17
1	79	11	10
2	80	7	13
3	82	5	13

[a] U2OS-GR(+) cells were cultured in the presence or absence of 100 nM DEX for the indicated period of time. DNA was stained with propidium iodide (see Materials and Methods), and the nuclear emitted fluorescence was measured in a FACScan flow cytometer.

and p27; the expression of these proteins is unaffected by DEX treatment in GR-expressing U2OS cells. We suggest that ligand-modulated transcriptional induction of p21 and p27 in SAOS2-GR(+) cells facilitates the GR-induced growth arrest. On the basis of these findings, we hypothesized that the selection of a primary cellular target by the activated GR is dictated by its ability to be either a transcriptional activator or a transcriptional repressor depending upon the cell-type-specific background and thus can be ultimately mapped to specific receptor domains necessary for these functions.

Growth inhibition by the GR in U2OS cells is mediated by its DNA binding domain. To determine which functional domains of GR are essential for its ability to cause G_1 arrest in U2OS cells, we generated stable lines ectopically expressing deletion and substitution derivatives of the receptor. The GR deletion mutant Δ4C1 lacks amino acids 70 through 300, which comprise the N-terminal transcriptional activation domain (Fig. 5A). The substitution mutant NLxC is a chimeric protein with the GR DNA binding domain replaced with that of the bacterial LexA DNA-binding protein (Fig. 5B) (21). This latter derivative has been shown in CV-1 cells to be fully competent for signal transduction and transcriptional activation at a LexA DNA binding site (21). Both receptor derivatives were subcloned into a pCMV-Neo^r expression vector and transfected into the U2OS cell line as described in Materials and Methods. The stable expression of these receptor derivatives in multiple neomycin-resistant clones was verified by immunoblotting with the receptor-specific monoclonal antibody BuGR2 (not shown). We also compared the level of expression of these derivatives to that of the wt receptor and found the level and integrity of the receptors to be similar (not shown). In addition, the transcriptional activity of the Δ4C1 deletion and the NLxC substitution mutants was examined by transiently transfecting a GRE- or Lex operator-linked chloramphenicol acetyltransferase reporter gene into U2OS cell lines stably expressing these GR derivatives. Consistent with the removal of the N-terminal activation domain, the transcriptional activity of the GRΔ4C1 mutant stably expressed in U2OS cells was reduced over fivefold relative to that of the wt receptor expressed at the same level in the same cell type (not shown). The NLxC chimera was able to activate transcription on a specific LexA reporter element in U2OS cells (not shown). To test these GR derivatives in a cell proliferation assay, two U2OS-Δ4C1 and two U2OS-NLxC clones were cultured in the presence or absence of 10^{-7} M DEX and cell growth kinetics were determined as described in Materials and Methods. Figure 5A demonstrates that the Δ4C1 receptor N-terminal deletion construct

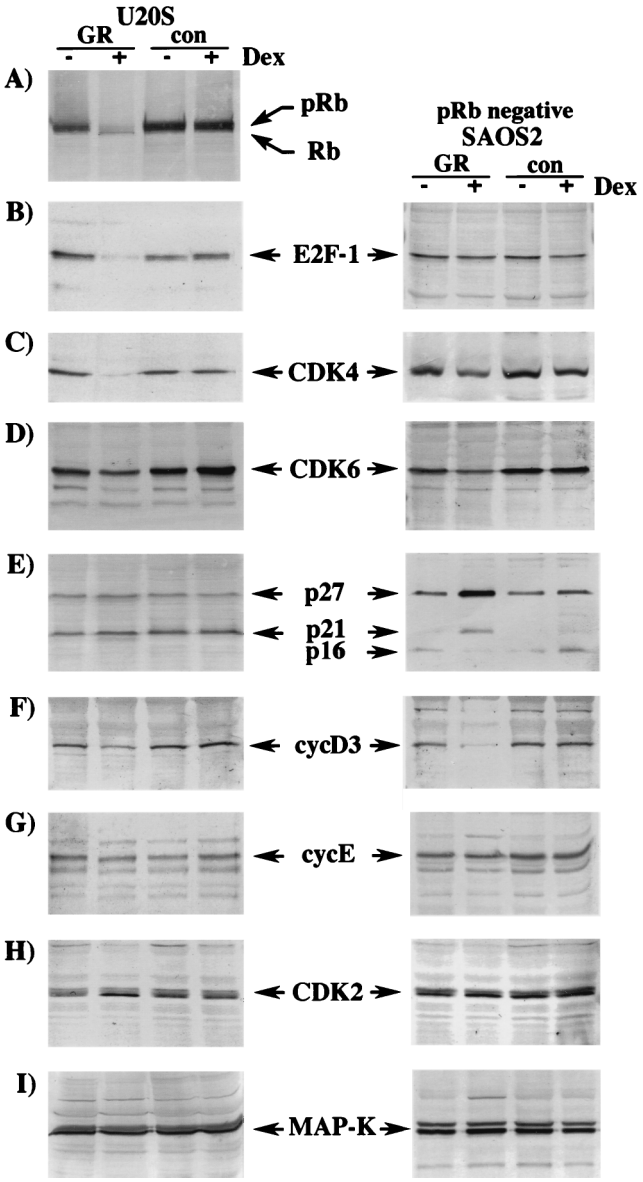

FIG. 3. Expression of cell-cycle-regulatory proteins in U2OS and SAOS2 GR-containing cells. U2OS and SAOS2 cells expressing GR (GR) or receptor-deficient control (con) cells were cultured in the absence (−) or presence (+) of 100 nM DEX for 3 days, and whole-cell lysates were prepared (Materials and Methods). Upon adjustment of the total protein concentration in each sample, the proteins were resolved on SDS–10 or 12.5% PAGE and transferred to Immobilon paper and the blots were probed with antibodies against Rb (A); E2F-1 (B); CDK4 (C); CDK6 (D); p27, p21, and p16 (E); cyclin D3 (F); cyclin E (G); CDK2 (H); and MAP-K (I). The antibodies used in each experiment are described in Materials and Methods. Each blot is representative of at least three independent experiments.

TABLE 2. Cell cycle distribution of SAOS2-GR(+) cells[a]

Treatment or day	% Fluorescence at cell cycle phase:		
	G_0/G_1	S	G_2/M
No hormone	47	25	28
3	69	12	19
5	72	9	19
7	68	13	19

[a] SAOS2-GR(+) cells were cultured in the presence or absence of 100 nM DEX for the indicated period of time. DNA was stained with propidium iodide, and cells were subjected to flow cytometry analysis (see Materials and Methods).

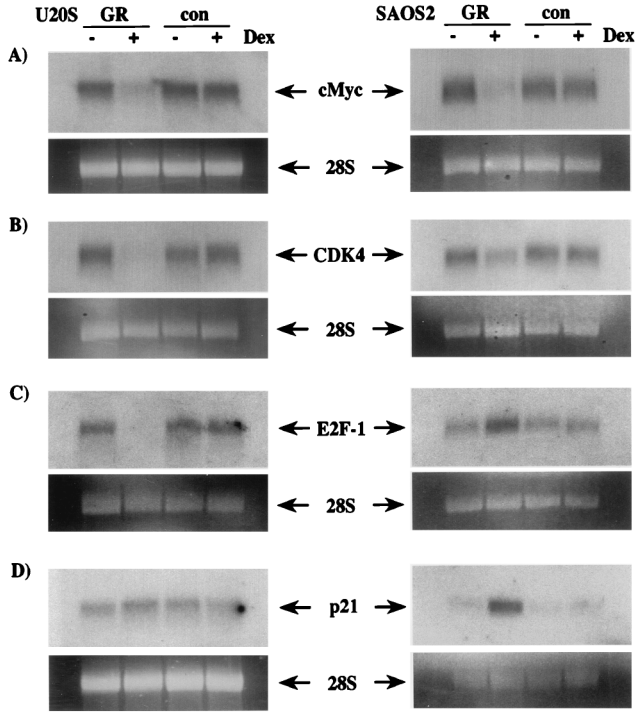

FIG. 4. Changes in steady-state mRNA levels of cell-cycle-regulatory proteins in U2OS and SAOS2 GR-expressing cells upon hormone treatment. Total RNA was extracted from U2OS and SAOS2 GR-expressing (GR) or control receptor-negative (con) cells cultured in the absence (−) or presence (+) of 100 nM DEX for 24 h. Equal amounts of RNA were separated on denaturing formaldehyde-agarose gels (see Materials and Methods), transferred to nitrocellulose, and hybridized to ^{32}P-labeled cDNA probes corresponding to c-Myc (A), CDK4 (B), E2F-1 (C), and p21 (D). Equal loading for each lane is demonstrated by ethidium bromide staining of 28S rRNA.

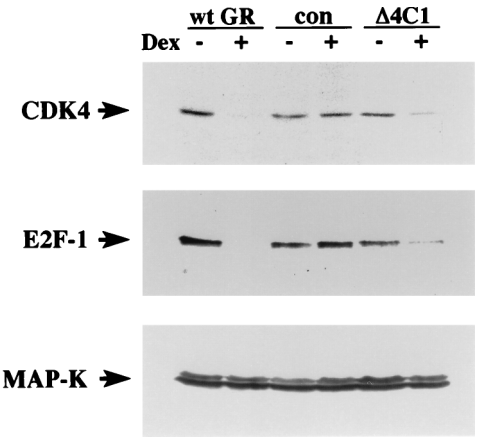

FIG. 6. Transcriptional activation-deficient Δ4C1 mutant effectively represses the protein levels of CDK4 and E2F-1. U2OS-GR(+) (wt GR), control U2OS-GR(−) (con), and U2OS-Δ4C1 (Δ4C1) cells were grown in the absence (−) or presence (+) of 100 nM DEX for 3 days. WCE were prepared (Materials and Methods), and the proteins were resolved on SDS–12.5% (for CDK4) or SDS–10% (for E2F-1 and MAP-K) PAGE for ECL-Western blot analysis. The membrane was probed with either anti-CDK4 rabbit polyclonal antibody (top panel) or anti-E2F-1 mouse monoclonal antibodies (middle panel), stripped, and reprobed with anti-MAP-K rabbit antiserum to control for equal loading (bottom panel). Similar results were obtained with four U2OS-Δ4C1 clones examined.

induces a ligand-dependent cell growth arrest as efficiently as does the wt receptor (compare to Fig. 1B), suggesting that the N-terminal transactivation domain is dispensable for the receptor's growth-inhibiting properties in U2OS cells. Morphological alterations identical to those seen in U2OS-GR(+) cells were also observed upon activation of this derivative (not shown). In contrast, the growth of cells ectopically expressing the NLxC chimeric protein was virtually unaffected by the DEX treatment, indicating that the DNA binding domain of the GR is necessary for GR-mediated cell cycle arrest (Fig. 5B).

To verify that the activated deletion mutant acts along the same pathway to induce the cell cycle arrest and morphological alterations as does the wt receptor, we compared the expression of CDK4 and E2F-1 proteins in U2OS cells carrying the wt GR and the Δ4C1 construct. Figure 6 illustrates that DEX

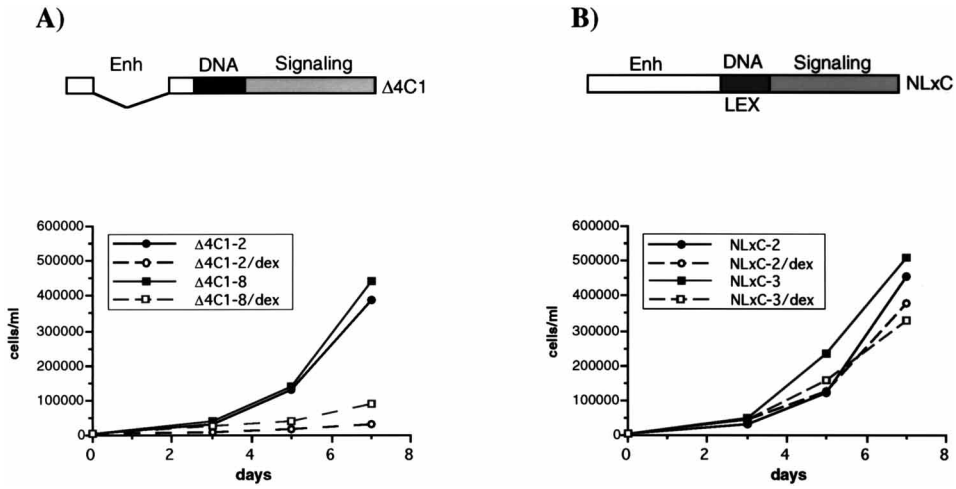

FIG. 5. Domains of GR mediating cell growth arrest in U2OS cells: effects of GR deletions and substitutions on cell proliferation. GR deletion (Δ4C1) and substitution (NLxC) derivatives were ectopically expressed in U2OS cells as described for Fig. 1A. Stable expression of both receptor mutants in multiple clones was verified by Western blotting (not shown), and the clones expressing each receptor derivative at levels comparable to that of a wt GR in U2OS-GR(+) cells were subjected to a cell proliferation assay as described for Fig. 1B. (A) A deletion of GR amino acids 70 through 300, which encompass the N-terminal transcriptional enhancement domain (clones Δ4C1 2 and 8), has no effect on GR's ability to induce cell cycle arrest in the presence of DEX. (B) A substitution of GR DNA binding domain with a heterologous LexA DNA binding domain (clones NLxC 2 and 3) abolishes receptor-mediated G_1 arrest, suggesting that the GR DNA binding domain is essential for GR-mediated cell cycle arrest.

treatment of U2OS-Δ4C1 cells depletes the cells of both CDK4 (top panel) and E2F-1 (middle panel) to a similar extent as observed in the U2OS-GR(+) clone. Similar results were obtained with four U2OS-Δ4C1 clones tested. Thus, deletion of the N-terminal activation domain yields a receptor with only a minimal ability to enhance transcription but fully capable of causing cell growth arrest and morphological changes via the same pathway utilized by the full-length protein. Together, these results argue that GR-dependent cell cycle arrest in U2OS cells is primarily mediated by the receptor's ability to repress, either directly or indirectly, the level and/or activity of growth-promoting factors, such as E2F-1, CDK4, and c-Myc.

GR N-terminal transcriptional enhancement domain is necessary for growth arrest in SAOS2 cells. Ligand-dependent changes in the expression of cell-cycle-regulatory proteins in U2OS and SAOS2 cells ectopically expressing GR indicate a striking difference in receptor signaling in these two cell lines. In U2OS-GR(+) cells, we observed a dramatic down-regulation of the gene products promoting the G_1-to-S-phase transition (E2F-1, CDK4, CDK6, and c-Myc), while in SAOS2-GR(+) cells the steady-state mRNA and protein levels of the growth-inhibitory factors p21 and p27 were significantly elevated upon ligand treatment. We hypothesized that the transcriptional activation by GR that appeared dispensable for the receptor's ability to induce G_1 arrest in U2OS cells may play an important role in inhibiting the proliferation of SAOS2 cells. To address this possibility, we generated SAOS2 cell lines expressing the Δ4C1 receptor deletion derivative that lacks the N-terminal transcriptional activation domain (Fig. 7A). For further studies, we used clones that expressed the Δ4C1 receptor deletion derivative at levels comparable to the levels of the full-length receptor in SAOS2-GR(+) cells (not shown). We then examined the effects of ligand activation of the Δ4C1 receptor mutant on cell proliferation. Strikingly, this receptor derivative failed to affect cell growth in two independent SAOS2-Δ4C1 cell lines (Fig. 7A), although the same construct induced complete ligand-dependent cell cycle arrest when expressed in U2OS cells (Fig. 5A).

We reasoned that the lack of cell cycle arrest in SAOS2 deletion clones would be consistent with the inability of the Δ4C1 construct to activate the expression of the CDIs p21 and p27. Figure 7B illustrates that, indeed, the dramatic ligand-dependent increase in the level of p21 and p27 proteins in SAOS2-GR(+) cells is not observed in the SAOS2-Δ4C1 clones expressing a transactivation-deficient mutant (compare lane 2 to lanes 6 and 8). This result strongly argues for the role of the receptor's N-terminal transcriptional activation domain in G_1 arrest in SAOS2 cells.

Induction of CDI expression in SAOS2-GR(+) cells involves direct transcriptional enhancement by the activated GR. The N-terminal transcriptional activation domain appears essential for both GR-mediated cell cycle arrest and enhanced expression of the CDIs p27 and p21 in SAOS2 cells. Since the changes in mRNA expression were analyzed after a 24-h hormone treatment, we could not exclude the possibility that receptor induces another protein, whose enhanced expression then leads to transcriptional activation of p27 and p21. To address this possibility, we first assessed the time course of p21 mRNA induction in SAOS2-GR(+) cells exposed to hormone. Figure 8A illustrates that a 3-h exposure to DEX is sufficient to induce maximal p21 expression, with no further changes in level of the message occurring upon longer treatment. Furthermore, pretreatment of cells with the protein synthesis inhibitor cycloheximide prior to a 2.5-h hormone treatment did not block GR-dependent induction of p21 mRNA (Fig. 8B), suggesting that transcriptional activation of p21 does not require translation of a putative GR-responsive intermediate. Thus, CDI induction by the activated receptor is a rapid, protein-synthesis-independent response, mediated by the N-terminal activation domain of GR.

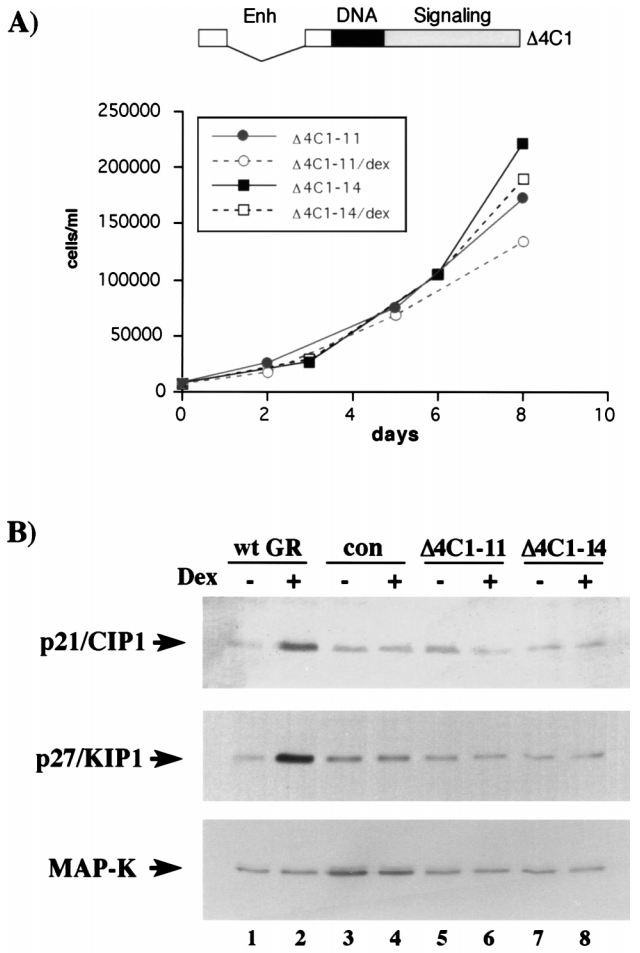

FIG. 7. Effects of Δ4C1 deletion on the GR's ability to inhibit cell proliferation in SAOS2 cells. (A) Cell proliferation assay with Δ4C1-expressing SAOS2 cells. The transcriptional activation-deficient Δ4C1 derivative was stably transfected into SAOS2 cells, and the level of expression of the deletion mutant in multiple clones was assessed by Western blotting (not shown). Two SAOS2-Δ4C1 clones (Δ4C1-11 and Δ4C1-14) were cultured in the absence (filled circles and filled squares, respectively) or presence (empty circles and empty squares, respectively) of 100 nM DEX, and the number of viable cells was determined on the indicated days as described for Fig. 1B. The graph represents one of three independent experiments. (B) Effect of Δ4C1 mutant activation on the expression of the CDIs p21 and p27. SAOS2-GR(+) cells (wt GR), SAOS2-GR(−) cells (con), and two SAOS2-Δ4C1 clones (Δ4C1-11 and Δ4C1-14) were grown in the absence (−) or presence (+) of 100 nM DEX for 3 days, and the level of p21/CIP1 (top panel) and p27/KIP1 (middle panel) expression was assessed by ECL-Western blotting. To control for identical loading in each lane, the membrane was stripped and reprobed with anti-MAP-K antibodies (bottom panel). The blot is one of three or more representative experiments.

GR-dependent activation of CDI expression extends to murine S49 lymphoma cells. We have demonstrated that transcriptional-activation-competent GR ectopically introduced into Rb-negative SAOS2 cells induces the expression of CDIs p21 and p27 and a G_1 cell cycle arrest. GR activation has also been linked to cell cycle arrest and apoptosis in cell lines of lymphoid origin, including human Jurkat cells and mouse S49 lymphoma cells.

Based on our findings, we hypothesized that induction of CDIs might be a critical step for cell cycle arrest in glucocor-

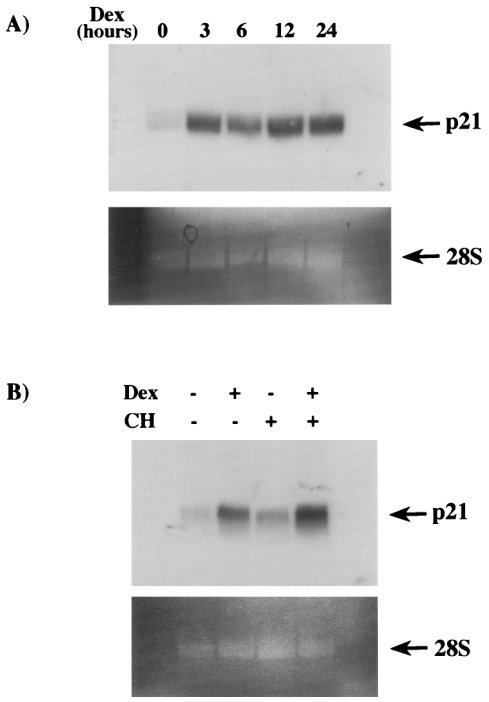

FIG. 8. Transcriptional induction of the CDI p21 mRNA in SAOS2 cells by the activated GR. (A) A time course of p21 mRNA expression. SAOS2-GR(+) cells were cultured in the presence of 100 nM DEX for 0, 3, 6, 12, and 24 h; total RNA was isolated and subjected to Northern blotting with a ^{32}P-labeled p21 cDNA as a probe as described in Materials and Methods. Equal loading for each lane is demonstrated by ethidium bromide staining of 28S rRNA. (B) The p21 mRNA induction is cycloheximide insensitive. SAOS2-GR(+) cells were incubated for 2.5 h in the presence or absence of 10 μg of cycloheximide (CH) per ml, followed by 100 nM DEX treatment for another 2.5 h where indicated. Total RNA was isolated and subjected to Northern blot analysis with p21 cDNA as a probe.

ticoid-sensitive cells, in which transcriptional activation by the GR appears essential, for example in S49 cells. Conversely, in cells undergoing growth arrest and apoptosis as a result of GR-mediated transcriptional repression, for instance in human Jurkat cells, no CDI activation will occur. Furthermore, GR-dependent induction of p21 and/or p27 would be observed in Rb-negative and not Rb-expressing cells.

To address these questions, we examined the expression of p27 and Rb proteins in mouse S49 lymphoma and human Jurkat T cells. Although we were unable to detect any p21 protein in S49 cells (see Discussion), the level of the p27 protein (and mRNA [not shown]) is significantly increased in S49 cells in response to DEX treatment (Fig. 9A). In mutant S49 cells that are glucocorticoid resistant due to the loss of GR, no increase in p27 level is observed upon hormone treatment (Fig. 9A). Significantly, S49 lymphocytes are devoid of Rb protein, like SAOS2 cells, whereas Jurkat cells produce Rb at the level comparable to that expressed in U2OS cells (Fig. 9B). To verify that the polyclonal antibodies used to detect Rb cross-react with mouse and human proteins with similar affinity, we tested them against bacterially expressed and purified mouse Rb and found the affinity to be similar (not shown). In addition, the same blot was reprobed with mouse-specific anti-Rb antibodies, and no Rb was detected in S49 cells (not shown).

Together, these findings suggest a role for cellular factors in determining the mechanism of GR-dependent inhibition of cell proliferation: in Rb-expressing cells, the repressive function of the receptor mediates growth inhibition, whereas in cells lacking Rb, the activation function of the receptor dictates growth arrest.

DISCUSSION

Distinct GR domains mediate cell cycle arrest in U2OS and SAOS2 cells. Glucocorticoids are potent antiproliferative

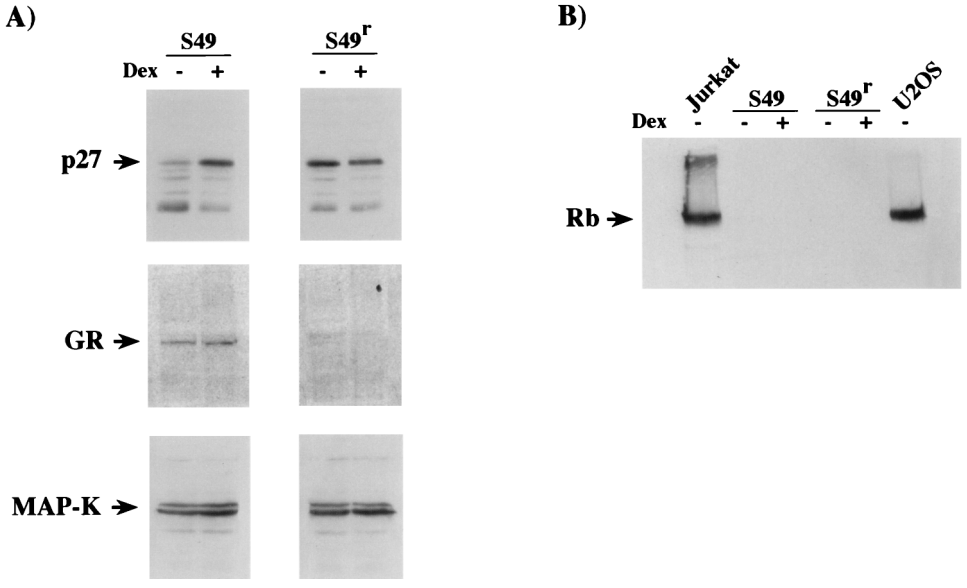

FIG. 9. GR-dependent induction of p27 in mouse S49 lymphoma cells. (A) DEX treatment induces the CDI p27 expression in S49 mouse lymphoma cells. Parental S49 cells and glucocorticoid-resistant S49^r cells were treated with 100 nM DEX overnight, and whole-cell lysates were prepared. Upon adjustment of the total protein concentration in each sample, the proteins were resolved on 10% PAGE, transferred to Immobilon paper, and probed with anti-p27 monoclonal antibody (top panel) as described in Materials and Methods. The blot was stripped and reprobed first with GR-specific monoclonal antibody (BuGR2) (middle panel) and then with polyclonal antiserum against MAP-K (bottom panel) to control for equal loading. (B) Rb expression in S49 and Jurkat cells. WCE from Jurkat, S49, S49^r, and U2OS cells were prepared as described above and tested for Rb expression with a rabbit polyclonal antiserum that cross-reacts with both human and mouse Rb. The ability of these antibodies to recognize mouse Rb was confirmed with recombinant, purified mouse Rb protein (not shown).

agents in multiple cell types. The growth-inhibitory effect of glucocorticoids is accomplished through the activated hormone-receptor complex which binds to specific genomic sites where it can activate or repress transcription. We have shown that ectopic expression and activation of GR in the human osteosarcoma cell lines U2OS and SAOS2 lead to ligand-dependent G_1 cell cycle arrest. Remarkably, we found that distinct receptor domains mediate growth arrest in these two cell lines. The receptor N-terminal transcriptional activation domain is required for cell cycle arrest in SAOS2 cells, whereas it is completely dispensable for growth arrest in U2OS cells. On the other hand, the zinc-finger region of the receptor, but not the N-terminal transcriptional activation domain, is necessary for G_1 arrest in U2OS-GR(+) cells. The zinc-finger region is involved in sequence-specific DNA binding as well as interaction with nonreceptor proteins to facilitate transcriptional repression (75). One simple interpretation of our findings is that, in SAOS2 cells, GR-mediated transcriptional activation is the dominant mode of receptor regulation underlying growth arrest, while receptor-mediated transcriptional repression is the mechanism of growth inhibition in U2OS cells. It is conceivable that, in cells expressing low levels of Rb, a combination of repression of mitogenic factors and activation of antimitogenic factors by GR may bring about growth arrest.

Cell-type-specific targets of GR action. Studies of the cell-cycle-regulatory proteins affected by GR activation also support our hypothesis whereby transcriptional activation and transcriptional repression mediate growth arrest in SAOS2 and U2OS cells, respectively. For example, in SAOS2-GR(+) cells, the antimitogenic CDI proteins p27 and p21 are induced by ligand treatment. Furthermore, when a mutant GR lacking the N-terminal transcriptional activation domain is expressed in these cells, induction of p21 and p27 is abolished and the cells do not undergo cell growth arrest. In contrast, GR in U2OS-GR(+) cells fails to induce p27 and p21 expression but instead represses multiple mitogenic factors, including cyclin D3, CDK4, and CDK6 as well as the transcriptional regulatory proteins E2F-1 and c-Myc. Deletion of the N-terminal transcriptional activation domain yields a derivative fully capable of inducing ligand-dependent cell growth arrest in U2OS cells and repressing the same mitogenic proteins as does the wt receptor.

It has been previously shown that, in certain murine and human lymphoid cells undergoing glucocorticoid-induced growth arrest and apoptosis, a variety of mitogenic factors are transcriptionally repressed. These genes include the cellular oncogenes c-*myc* (48, 63), c-Ki-*ras*, and c-*myb* (16) as well as the cell-cycle-regulatory proteins cyclin D3 (48) and CDK4 (49). Using GR mutations that uncouple transcriptional activation from repression, Helmberg et al. suggest that the repression of AP-1 activity by the receptor is sufficient for glucocorticoid-induced cell cycle arrest and programmed cell death in human Jurkat cells (23). In genetic reconstitution experiments using the glucocorticoid-sensitive murine lymphoma cell line P1798, Rhee et al. demonstrated that c-*myc* and cyclin D3 undergo ligand-dependent transcriptional repression and that ectopic expression of both proteins, but of neither one individually, prevented glucocorticoid-induced cell cycle arrest (48). In S49 mouse lymphoma cells, transcriptional activation by GR appears to be the critical event leading to growth arrest, and recently, a number of GR-induced target genes have been identified (7, 15). Our findings suggest that, as in Rb-negative SAOS2 cells, GR activation in S49 cells leads to the induction of the antimitogenic protein p27. Attempts to detect either basal or hormone-induced expression of p21 protein in S49 cells by immunoblotting have thus far been negative. We do not know whether this lack of expression is due to a deletion of the p21 gene, to a lack of the factors regulating its expression, or to the possibility that the level of p21 is below the sensitivity of our detection methods. If p21 is indeed not expressed in S49 cells, then it is consistent with the recent findings that forced expression of p21 blocks apoptosis in myocytes induced by mitogen deprivation (67). It is tempting to speculate that the lack of p21 in S49 cells may facilitate apoptosis. If this were the case, it seems likely that p27 fulfills the role of the growth-inhibitory proteins induced by glucocorticoid treatment in S49 cells alone or in conjunction with additional hormone-induced proteins. Indeed, the identification of early glucocorticoid-responsive genes in S49 cells (7) may provide potential candidates that cooperate with p27 to induce growth arrest. The notion that p21 protects against apoptosis is also consistent with our preliminary findings that, in SAOS2-GR(+) cells, in which this protein is hormone induced, cells fail to undergo glucocorticoid-induced apoptosis, whereas U2OS-GR(+) cells undergo apoptosis and no p21 induction is observed. Clearly, the involvement of p21 and p27 in GR-mediated growth arrest, apoptosis, and survival is complex and will require further investigation.

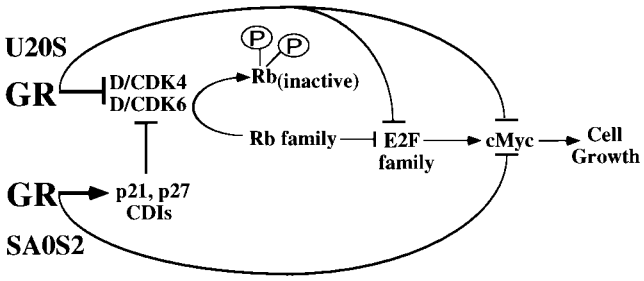

FIG. 10. A model for GR-mediated cell cycle arrest. GR activation in U2OS-GR(+) cells represses the expression of G_1-acting cyclin-CDK complexes including D-type cyclins and their catalytic partners CDK4 and CDK6, which results in hypophosphorylation of Rb protein. The active form of Rb binds to and inactivates E2F-1, thereby preventing the transcription of multiple E2F-responsive genes including E2F-1 itself and c-*myc*. In SAOS2-GR(+) cells, the activated GR enhances transcription of CDIs p21 and p27. Since Rb is not expressed in these cells, there might exist an alternative pathway involving other pocket proteins (p107 and p130) and E2F family members. This model appears to extend to glucocorticoid-sensitive lymphocyte cells. Murine S49 lymphoma cells, like SAOS2 cells, lack Rb and employ transcriptional activation by GR to facilitate growth arrest, perhaps through the induction of CDI expression. Jurkat cells, like U2OS cells, contain Rb and utilize transcriptional repression by the receptor to bring about growth arrest.

A model for glucocorticoid-induced cell cycle arrest. Activation of ectopically expressed GR in U2OS and SAOS2 human osteosarcoma cells leads to a cell cycle arrest at G_1, wherein receptor activation and cell growth arrest are coupled to cell-type-specific changes in the pattern of gene expression. The likely mechanism of receptor-mediated G_1 cell cycle arrest in U2OS cells involves transcriptional repression of mitogenic factors, such as CDK4, CDK6, and cyclin D3, which results in hypophosphorylation of Rb protein (Fig. 10). This hypophosphorylated form of Rb sequesters E2F-1 in a transcriptionally inactive multiprotein complex, thereby preventing the expression of the genes essential for the G_1-to-S-phase transition. As a consequence, or perhaps through more direct receptor action, the expression of transcription factors E2F-1 and c-*myc* is also repressed. E2F binding sites in the promoters of both the E2F-1 (43) and c-*myc* (38) genes as well as the transcriptional enhancement of these genes by E2F-1 (26, 45) have been shown previously.

In SAOS2-GR(+) cells, hormone-activated GR appears to

utilize a different mechanism, i.e., activation of CDIs p21 and p27, to achieve growth arrest. These proteins inhibit multiple CDKs, including cycD3/CDK4 and cycD3/CDK6, the only D-type complexes in SAOS2 cells. Given that Rb is not expressed in this cell line, there may exist an alternative mechanism of growth arrest involving CDIs and possibly other members of both Rb and E2F protein families, which could account for the repression of c-*myc*, and receptor-induced cell cycle arrest (Fig. 10). This model appears to apply to murine S49 lymphoma cells that express endogenous GR. Like SAOS2-GR(+) cells, S49 cells require the receptor N-terminal activation domain for growth arrest and apoptosis, lack Rb, and induce p27 expression upon GR activation.

CDIs act as central targets for differentiation-inducing factors (61). For example, inducers of myeloid cell differentiation activate the expression of p21 and p27 (29, 68). The p21 promoter contains multiple response elements for several differentiation signals (8, 77), which are scattered throughout the promoter and in some cases overlap to create synergistic responses (12). Furthermore, in an elegant set of experiments, Liu et al. have observed binding to and activation of the p21 promoter by the vitamin D receptor in U937 cells that undergo vitamin D-induced cell cycle arrest (34). It appears likely that, in addition to the vitamin D receptor, p21 gene expression is also regulated by GR and that this regulation is cell type specific. Although we are continuing to determine whether the action of GR on the target genes examined is direct, we cannot exclude the possibility that GR-dependent growth arrest of these osteosarcoma cells is due to transcriptional regulation of a gene other than those examined.

At present we do not know whether Rb status alone determines the receptor domains required for growth arrest or is simply a marker. We would like to determine whether forced expression of Rb in SAOS2-GR(+) cells would alter the receptor domain required for growth arrest. Unfortunately, ectopic expression of Rb in SAOS2 cells leads to a G_1 cell cycle arrest (17), and since GR transcriptional enhancement is regulated throughout the cell cycle (27), interpretation of the results would be problematic. Nevertheless, we are continuing our efforts to understand these cell-type-specific requirements for the GR domains and the target genes that facilitate growth arrest, by extending our analysis to other Rb-deficient cell lines, including C-33A human cervical carcinoma cells and primary embryo fibroblasts from Rb knockout mice (28).

Potential mechanisms underlying the cell-type-specific differences of receptor action. The observed differences in the receptor regions and cellular targets governing cell cycle arrest in U2OS and SAOS2 cells are likely to reflect differences in the regulatory proteins resident in these cell lines, which in turn may predetermine whether it is activation or repression of genes by the receptor that halts the cell cycle progression. Which factors are responsible for these cell-type-specific differences? One plausible explanation is the presence of an accessory transcriptional regulatory protein in Rb-deficient cells which is capable of synergizing with GR at particular promoters to enhance transcription of a target gene. This factor might productively interact with the N-terminal region of the receptor to induce transcriptional activation of the p21 and p27 promoters in SAOS2 cells. We suggest that this putative activity is not p53, a known activator of p21 (35), since the expression of p53 protein is not induced by receptor activation in SAOS2-GR(+) cells (not shown). Alternatively, it may not be the presence or absence of a particular cellular factor in a given cell line that is important, but rather the relative abundance of ubiquitous factors, such as c-Jun and c-Fos. Previous findings from the Yamamoto laboratory suggest that GR can switch from a transcriptional activator to a transcriptional repressor depending upon the combination of regulatory factors present in a certain cell line (73). Alterations in the levels or type of AP-1 family members may instruct the receptor to activate or inhibit transcription at particular promoters. In preliminary studies, the level of c-Jun is not affected by receptor activation in either cell line (not shown). Another possibility is that the chromatin structure surrounding regulatory regions of certain promoters, such as p21 or p27, may differ in the two cell types, making them more accessible to transcription factor binding and activation. Interestingly, Rb-deficient mouse primary embryo fibroblasts exhibit a more relaxed chromatin structure by virtue of increased histone H1 phosphorylation (24). This open chromatin configuration may enable GR either alone or in concert with other regulatory factors to activate transcription from certain promoters more effectively in SAOS2 cells. Similarly, alterations in composition or level of chromatin remodeling factors, such as the SWI/SNF protein complex previously shown to associate with GR (39, 59, 76), may affect the receptor's ability to penetrate chromatin and activate transcription. Finally, the receptor may exhibit a distinct pattern or enhanced level of phosphorylation, owing to differential expression of protein kinases or phosphatases in the two cell lines, which in turn may contribute to differences in receptor transcriptional regulation. In support of this idea, activity of the CDK cyclin E/CDK2 is elevated in Rb-deficient mouse embryo fibroblasts (24), and this kinase has recently been shown to phosphorylate the N-terminal transcriptional activation domain of the receptor and increase GR transcriptional enhancement (32).

Differences in the GR domains and the target genes facilitating growth arrest may also result from an inhibitory factor present in U2OS cells that prevents the N-terminal transcriptional activation domain from functioning. Factors including Rb and mdm2, which inhibit E2F- and p53-mediated transcriptional activation, respectively, are expressed in U2OS, but not in SAOS2, cells and may be potential candidates for such an inhibitor, although this remains to be tested. One or more of these factors may account for the distinct mechanisms of GR-mediated regulation of cell cycle progression.

Our findings provide a unifying view of ligand-induced growth arrest that accommodates differences in the receptor domains and modes of transcriptional regulation displayed by GR in different cell contexts. Thus, depending on the composition of regulatory proteins resident in a particular cell type, the receptor adopts an activating or repressing posture to bring about cell cycle arrest.

Implications for glucocorticoid use in clinical oncology. Glucocorticoids play a significant role in the treatment of many types of cancer (5, 60), including lymphoproliferative disorders (20, 31) and breast cancer (10), although their mode of action is unclear. Our study provides a molecular basis for the growth-inhibitory effects of glucocorticoids through the cell-type-specific repression or induction of growth-promoting or growth-inhibitory factors. Our results suggest that glucocorticoid treatment could reverse the growth advantage conferred on cells through the amplification of specific CDKs or by the loss of certain CDIs. Thus, malignancies that arise via overexpression of cyclin D3, CDK4, or CDK6 in Rb-expressing cells may be excellent candidates for glucocorticoid therapy. Furthermore, in cells lacking Rb, cell growth may be inhibited by the GR-mediated induction of the antimitogenic factors p27 and p21. Ongoing efforts to understand the mechanism of glucocorticoid-dependent cell growth inhibition will provide the basis for more rational approaches to the therapeutic use of glucocorticoids in cancer treatment.

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