Glucocorticoids Inhibit Developmental Stage-specific Osteoblast Cell Cycle

DISSOCIATION OF CYCLIN A-CYCLIN-DEPENDENT KINASE 2 FROM E2F4-p130 COMPLEXES*

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Unique cell cycle control is instituted in confluent osteoblast cultures, driving growth to high density. The postconfluent dividing cells share features with cells that normally exit the cell cycle; p27kip1 is increased, p21waf1ip1 is decreased, free E2F DNA binding activity is reduced, and E2F4 is primarily nuclear. E2F4-p130 becomes the predominant E2F-pocket complex formed on E2F sites, but, unlike the complex that typifies resting cells, cyclin A and CDK2 are also present. Administration of dexamethasone at this, but not earlier stages, results in reduction of cyclin A and CDK2 levels with a parallel decrease in the associated kinase activity, dissociation of cyclin A-CDK2 from the E2F4-p130 complexes, and inhibition of G1/S transition. The glucocorticoid-mediated cell cycle attenuation is also accompanied by, but not attributable to, increased p27kip1 and decreased p21waf1ip1 levels. The attenuation of osteoblast growth to high density by dexamethasone is associated with severe impairment of mineralized extracellular matrix formation, unless treatment commences in cultures that have already grown to high density. Both the antimitotic and the antiproliferative effects are reversible, and both are antagonized by RU486. Thus, glucocorticoids induce premature attenuation of the osteoblast cell cycle, possibly contributing to the osteoporosis induced by these drugs in vivo.

Glucocorticoids (GC),1 widely used as immunosuppressive and anti-inflammatory drugs, cause bone loss and increased fracture risk (reviewed in Ref. 1). Suggested mechanisms contributing to GC-induced osteoporosis include decreased osteoblastic bone formation, increased osteoclastic resorption, impaired intestinal calcium absorption, and decreased renal calcium reabsorption (reviewed in Refs. 2 and 3). Among these, impairment of osteoblast function, probably the main single contributor to GC-induced bone loss (4), is poorly understood. It may involve both indirect mechanisms, such as gonadal insufficiency and impaired hematopoiesis (reviewed in Refs. 2, 3, and 5), and direct, cell-autonomous effects, such as (i) induction of apoptosis (6), (ii) inhibition of type I collagen (7–9) and alkaline phosphatase (10, 11) gene expression, and (iii) decreased activity of growth factors acting in bone in an autocrine/paracrine fashion, in particular IGF-1 (12–14). Inhibition of osteoblast proliferation was also suggested to contribute to GC-induced osteoporosis (15, 16). Notably, the inhibitory effects of GC on osteoblasts occur at pharmacological concentrations and should not be confused with the positive effects observed at physiological concentrations (for a review, see Ref. 17).

GC are antimitogenic in several cell types. Remarkably, however, this effect is mediated via diverse mechanisms. For example, inhibition of lymphoid cell proliferation, which partly accounts for the anti-inflammatory property of GC, is mediated by a decrease in the levels of G1 cyclin and cyclin-dependent kinases (CDKs), in particular cyclin D3 and CDK4 (18–20), as well as c-Myc (21, 22). By contrast, in both hepatoma and lung alveolar cells, GC-induced cell cycle arrest has been attributed to induction of the CDK inhibitor (CDI) p21 (23–25). GC-induced osteoblast cell cycle arrest has been recently addressed in two osteosarcoma cell lines (26). In Rb- and p53-deficient SAOS2 cells, GC-induced cell cycle arrest involves up-regulation of the CDIs p21 and p27, whereas in U2OS osteosarcoma cells this same phenotype is mediated by repression of CDK4, CDK6, cyclin D, c-Myc, and E2F-1, all of which are positive regulators of the cell cycle. Mechanisms by which GC inhibit nontransformed osteoblast cell cycle progression have not been studied in depth.

Abrogation of osteoblast cell cycle progression may contribute to impaired bone formation in two ways. First, cell proliferation is simply required to provide enough cells capable of new bone formation. Second, the cell cycle may have additional, more specific roles in phenotype development, similar to the clonal expansion necessary for adipocyte differentiation (27, 28). Osteoblast differentiation in vitro follows a multistep program, microscopically typified by (i) a proliferation period, during which the cells form a monolayer; (ii) matrix maturation, morphologically characterized by cell condensation, and (iii) the formation of nodules, in which mineral is then deposited (29–31). We hypothesized that the cell condensation, which accompanies matrix maturation, reflects a unique proliferation phase required for phenotype progression. Indeed, this report demonstrates that in confluent MC3T3-E1 osteoblastic cultures, a unique cell cycle control is instituted, which is mech-
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anitically different from that operative in preconfluent cultures and is sensitive to glucocorticoids. Furthermore, we provide evidence that GC-mediated inhibition of the osteoblast persistent cell cycle is tightly linked to the inhibitory effect of GC on terminal differentiation, i.e. formation of mineralized extracellular matrix.

MATERIALS AND METHODS

Cell Culture—Like other osteoblastic cell lines, MC3T3-E1 cells are phenotypically heterogeneous (32). We therefore isolated 10 MC3T3-E1 subclones and screened those for calcium deposition in the absence and presence of the synthetic glucocorticoid dexamethasone (DEX) at 100–1000 nM. Although mineralization in most of the subclones was strongly inhibited by 100–1000 nM, we continued the study with one subclone, which reproducibly exhibited progressive extracellular calcium deposition starting on day 10 after plating. Stock plates were maintained in α-minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin and split every 4–7 days. To support differentiation, the medium was also supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate as described (33).

Flow Cytometry—Cell cycle analysis was performed according to Darzynkiewicz et al. (34). Briefly, cells were lightly trypsinized, resuspended in Hanks’ buffer, fixed in cold 70% ethanol, and stored at −20 °C. Cells were resuspended in 1 ml of Hanks’ buffer containing 5% fetal bovine serum and one-third the volume of 80% glycerol and clarified by brief centrifugation. Cells were treated with 10 μg/ml propidium iodide and 5 Kunitz units of DNase-free RNase A. The percentage of cells in G1, S, and G2 was determined using an EPICS® Profile Analyzer.

Transfection and Luciferase Assays—For transient transfection assays, MC3T3-E1 cells were plated in six-well plates (80,000 cells/well), and the calcium phosphate co-precipitation method of Chen and Okayama (35) was employed with 5 μg of Qiagen-purified plasmid DNA. The cells were harvested on the indicated days, always 24 h after medium change, and lysed in “reporter lysis buffer” (Promega). Luciferase activity was determined using a microtriter plate luminometer (MLX Dynex Technologies) and protein concentration determined using the Micro BCA protein assay reagent kit (Pierce). Reporter constructs contained the luciferase gene driven by the mouse mammary tumor virus (MXTV) promoter (a kind gift from Dr. Ron Evans, La Jolla, CA), the Id2 promoter (36), the p21 promoter (37), and an artificial promoter, G13, containing 13 binding sites for p53 (37).

Nuclear, Cytoplasmic, and Whole Cell Extracts—Whole cell lysates were prepared using 0.5% (v/v) Nonidet P-40 buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl 20 μg/ml tissucolysinidum chloromethyl ketone, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, 10 mM NaF, and 0.1 mM Na3VO4. Lysates were passed through a 27-gauge needle, centrifuged at 13,000 × g for 20 min, and the supernatant was stored at −80 °C. Nuclear and cytoplasmic extracts were prepared essentially according to Verona et al. (38). Cells were briefly trypsinized and washed with phosphate-buffered saline. Cell pellets were resuspended, by flicking the tube, in two packed cell volumes of hypotonic buffer containing 10 mM HEPES (pH 7.5), 10 mM KCl, 3 mM MgCl2, 0.05% Nonidet P-40, 1 mM EDTA (pH 8), 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiorthiolum, 10 μM aprotinin, 10 μg/ml leupeptin, 10 mM NaF, and 0.1 mM Na3VO4. Cells were left to swell on ice for 10 min and then vortexed for 10 s and spun at 500 × g for 5 min. The supernatant, containing the cytoplasmic lysate, was supplemented with one-third the volume of 80% glycerol and clarified by centrifugation at 20,000 × g for 30 min. The nuclear pellet was washed twice in hypotonic buffer and lysed in two pellet volumes of lysate buffer containing 10 mM HEPES (pH 7.4), 0.5% KC1, 5 mM MgCl2, 28% glycerol, and protease and phosphatase inhibitors as above. The nuclear extracts were centrifuged at 20,000 × g for 1 h to remove cell debris. Protein concentration was determined using the Micro BCA protein assay reagent kit (Pierce).

Western Analysis—Between 60 and 100 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a 0.2-μm nitrocellulose membrane using Mini Trans-Blot Transfer Cell (Bio-Rad), and immunodetection was performed using ECL (Amersham Pharmacia Biotech) according to the manufacturer’s recommendations, followed by exposure of the membranes to film. The fluorogram was quantitated by densitometry of the film using the Alphalmager 2000 system (Alpha Innotech Corp.) Results from one representative experiment out of three independent experiments are shown for each analyte.

Retrovirus-mediated p27 Overexpression—The murine p27 cDNA (45) was amplified by polymerase chain reaction using Pfu polymerase (Stratagene). The forward primer, 5′-GAGCGTCGGATCCAAGAAAGAGGGGCCCAG, contained an SfuI recognition site (underlined), and the reverse primer, 5′-ATAAGCGGCGGTTCACGTCGAG, contained a NotI recognition site (underlined). The 602-base pair amplicon was digested with SfuI and NotI and cloned into the retroviral replication-competent vector pZAPd, downstream of the encephalomyocarditis virus internal ribosomal entry site (IRES) (2). 293 D probable pZAPd-p27. In preliminary experiments with pZAPd-emd, which contains the eukaryotic marker, the Parksd Biosciences (the green fluorescent protein (GFP) under the control of the encephalomyocarditis virus IRES, calcium phosphate transfection (35) of MC3T3-E1 cells resulted in nearly complete transduction of the entire cell population (see Fig. 4B), which was confirmed histochemically by Alizarin red staining of 70% ethanol-fixed cultures as described previously (41). For cyclin A, cyclin E, and CDK2 immunoprecipitation, 100 μg of cell lysate was incubated with 1 μg of primary antibody, followed by 20 μl of Ag-agarose bead suspension. For cyclin D1, 500 μg of cell lysate was immunoprecipitated with 3 μg of primary antibody. The immunoprecipitates were washed twice with 0.5% (v/v) Nonidet P-40 buffer as above and once with kinase buffer (41) containing protease and phosphatase inhibitors as above. Immunocomplexes were subjected to kinase assay in the presence of [γ-32P]ATP and either histone H1 (for cyclin A, cyclin E, and CDK2) or PRβ (residues 789–921) (for cyclin D1) as substrate, followed by SDS-polyacrylamide gel electrophoresis (10% gel containing 5% glycerol) and autoradiography. For kinase inhibition assays (41, 42), immunocomplexes were resuspended in 0.5% (v/v) Nonidet P-40 buffer as above boiled for 5 min, and centrifuged, and the supernatant was added to active extracts prior to immunoprecipitation and kinase assay.

Extracellular Matrix Mineralization—Calcium deposition was demonstrated histochemically by Alizarin red staining of 70% ethanol-fixed cultures as described previously. For quantitation of calcium accumulation, cell layers were initially scraped in saline solution containing 10 mM Tris-HCl (pH 7.8) and 0.2% Triton X-100 and centrifuged, and an aliquot was removed for protein determination using the BCA protein assay reagent kit (Pierce). HCl was then added to 0.5M, and the dissolved calcium was quantitated based on the light absorbance of complexes formed between calcium ions and o-cresolphthalein, using Sigma Procedure no. 587. Results are hence expressed as calcium per protein.

The materials and culture medium and Hanks’ buffer were purchased from Life Technologies, Inc. Fetal bovine serum was from Omega Scientific (Tarzana, CA). Dexamethasone, RU486, RNAse A, protease inhibitors, salmon sperm DNA, mouse IgG, and rabbit IgG were purchased from Sigma. NaF and Na3VO4 were from Aldrich. Histone H1 was purchased from Roche Diagnostics, and A/G beads, pBR (residues 769–921) (SC-4112), and antibodies against p130, p107, p27, cyclins A, D1, D2, D3, and E, CDK2, and CDK4 (SC-317, SC-250, SC-528, SC-596, SC-450, SC-593, SC-182, SC-481, SC-163, and SC-260, respectively) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to human p27 (Santa Cruz Research) were used at 2 μg/ml, and Anti p27 (Santa Cruz Research) was used at 2 μg/ml. Anti cyclin A (for supershift analysis), E2F2 (LLF4–2), and p27 were generous gifts from Dr. M. Pagano (New York University), Dr. J. Lees (Massachusetts Institute for Technology), and Dr. T. Fung (Children’s Hospital, Los Angeles), respectively.

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RESULTS

GC Inhibit Cell Cycle Progression in Postconfluent MC3T3-E1 Osteoblasts

To address the mechanism underlying the GC-mediated attenuation of cell cycle progression in postconfluent MC3T3-E1 cells, we initially determined the effect of DEX on expression of the CDIs p21 and p27, which play a role in the antimitogenic activity of GC in hepatoma, lung alveolar, and osteosarcoma cells (23–26). As shown in Fig. 4A, the level of p21 is decreased, not increased, in DEX-treated MC3T3-E1 cells, thus ruling out this CDI as the mediator of the GC effect.

In contrast to p21, Fig. 4A demonstrates a >2-fold DEX-induced increase in p27 levels (2.3 ± 0.2-fold in three independent experiments), which could contribute to the inhibition of G1/S transition in DEX-treated postconfluent MC3T3-E1 cells. This possibility was further addressed by overexpression of p27. We employed the replication-competent retroviral vector pZAp, which comprises the Moloney murine leukemia virus genome into which was inserted the IRES from the encephalomyocarditis virus (Fig. 4B).

As revealed by flow cytometry analysis for GFP expression, 9% of the cells in the infected culture expressed GFP by day 8 postinfection, and this increased to 99.1% within the following 6 additional days after passing (Fig. 4B). Next, p27 cDNA was cloned in place of GFP, and the expression of p27 in infected MC3T3-E1 cultures was evaluated by Western analysis. As shown in Fig. 4C (top), the pZAp-d-p27-infected cells expressed p27 at levels severalfold higher than those observed in control pZAp-d-emd-infected cells. These levels are also higher than those of DEX-treated (Fig. 4A) or serum-starved cells, used as control (Fig. 4C, top).

If p27 plays a pivotal role in the DEX-induced growth inhibition, then this may be mimicked by postconfluent p27-overexpressing cells in the absence of the steroid. However, as demonstrated by propidium iodide staining and flow cytometry analysis, the cell cycle profile of postconfluent p27-enriched cells was only marginally affected, whereas each of the DEX-treated or serum-deprived cells, serving as positive controls, exhibited the expected decrease in the percentage of cells at the S/G2/M phases (Fig. 4C).

Dissociation of Cyclin A and CDK2 from E2F4-p130 Complexes in GC-treated Osteoblasts Growing to High Density—E2F proteins play a pivotal role in the transcriptional regulation of numerous cell cycle regulatory genes (reviewed in Ref. 47). To address whether the osteoblast developmental stage-specific DEX-mediated G1/S attenuation resulted from alterations in E2F complexes, electrophoretic mobility shift assay was performed with nuclear extracts of nontreated and GC-treated MC3T3-E1 cells, either growing to confluency (day 3) or to high density (day 6). Similar to a variety of other cell types (see, for example, Refs. 48–50) the E2F binding activity in MC3T3-E1 cells consists of a number of fast migrating free E2F complexes and a cluster of slow migrating E2F-pocket complexes (Fig. 5A). As compared with the preconfluent cells (day 3), a prominent reduction in the free E2F complexes is observed...
in the postconfluent cultures. There are also significant alterations in the composition of the E2F-pocket cluster (Fig. 5A; see below). DEX did not affect the E2F binding activities in the preconfluent stage (Fig. 5A; see Fig. 5, B and C). However, at the postconfluent stage (day 6), DEX decreased the free E2F-DNA binding activity, and the E2F-pocket cluster was significantly altered, exhibiting a new, faster migrating complex. As opposed to the nuclear fraction, cytoplasmic E2F binding activity in the postconfluent cultures was not affected by DEX (Fig. 5A, Cyt). The cytoplasmic activity consisted of one major free E2F complex and a light E2F-pocket, the latter co-migrating with that seen in the DEX-treated postconfluent nuclear extracts (Fig. 5A).

Competition analyses were performed to characterize the protein-DNA complexes, initially in preconfluent cultures (Fig. 5, B and C). With the exception of the fastest migrating band, all of the complexes exhibited DNA sequence-specific binding (compare lanes wt and mt). Supershift analysis with antibodies to E2F4, p107, and p130 suggest that in the preconfluent stage, the E2F complexes are similar between nontreated (Fig. 5B) and DEX-treated (C) cultures. In both cases, the slowest migrating free E2F complex was identified as E2F4, since anti-E2F4 antibodies attenuated its migration to the position marked by the closed circle (Fig. 5, B and C; see Fig. 6). E2F4 is also present in one of two E2F-pocket complexes (arrow), which is supershifted to the position marked by an open circle. The pocket component of this complex (arrow) is mainly p130, because anti-p130 antibodies block its formation. The composition of the other E2F-pocket complex (arrowhead) has not been determined. The light supershifted band observed with anti-p107 antibodies indicates that this protein is also represented in the preconfluent E2F-pocket complexes from both nontreated and DEX-treated cells (Fig. 5, B and C).

The abundance of E2F4-p130 complexes in preconfluent actively proliferating MC3T3-E1 cells was unexpected, because E2F4-p130 complexes typically form in cells that undergo growth arrest, replacing E2F4-p107 complexes (51). To ascertain the expression and nuclear localization of E2F4 and p130 in proliferating MC3T3-E1 cells, the nuclear and cytoplasmic fractions were subjected to Western analysis. As shown in Fig. 5D, both E2F4 and p130 are present abundantly in MC3T3-E1 cell nuclei, regardless of developmental stage or DEX treatment. Interestingly, E2F4 cellular localization is developmentally regulated, exhibiting an equal contribution to nuclear and cytoplasmic proteins until confluency but a clear nuclear preference thereafter (Fig. 5D).

Analysis of the E2F complexes in postconfluent untreated MC3T3-E1 cells is presented in Fig. 6A. The predominant E2F-pocket complex (arrow) consists of E2F4 and p130 as antibodies against each of these proteins blocked or supershifted the...
majority of the E2F-pocket cluster. This complex thus appears to be identical to that of preconfluent cultures (compare with Fig. 5, B and C, arrow), based on co-migration (Fig. 5A) and supershift analyses. In contrast, the postconfluent cultures seem to have lost the other E2F-pocket complex present in the preconfluent cells (compare with Fig. 5, B and C, arrowhead).

As shown in Fig. 5A, postconfluent DEX-treated cultures exhibit one major E2F-pocket complex but with faster migration compared with that of nontreated cells. Fig. 6D demonstrates that, similar to the major E2F-pocket complex of the postconfluent nontreated cells (Fig. 6A, arrow), the faster migrating complex of the treated cells (white arrow) also consists of E2F4 and p130. Therefore, the migration difference between the E2F4-p130 complexes in postconfluent treated versus nontreated cells is probably accounted for by the loss of some additional component(s). These could be cyclin-CDK2 complexes, which typically bind to the inhibitory E2F-pocket complexes in proliferating cells, resulting in release of the pocket protein to yield free E2F (52).

To address the hypothesis that DEX inhibits postconfluent MC3T3-E1 cell cycle progression by dissociating cyclin-CDK2 from E2F4-p130 complexes, supershift analysis was performed with antibodies to CDK2, cyclin A, and cyclin E. Indeed, CDK2 antibodies strongly affected the E2F4-p130 complex (arrow) in nontreated postconfluent cultures (Fig. 6C), while no effect was observed on the E2F4-p130 complex of postconfluent DEX-treated cells (Fig. 6D, white arrow). Supershift analysis of the nontreated cells with CDK2 in combination with either E2F4 or p130 antibodies demonstrated a further shift compared with that observed with CDK2 antibodies alone, indicating co-existence of the three proteins in the same complex (Fig. 6C). The E2F4-p130 complex of the preconfluent cells (Fig. 5, B and C, arrow) also contains cyclin-CDK2 (data not shown).

We next addressed the identity of the cyclin component, responsible for tethering (and probably activating) CDK2 to the E2F4-p130 complex of postconfluent nontreated cells. To this end, we added cyclin A antibodies to the supershift reaction that already contained anti E2F4 antibodies. Fig. 6C (right panel) demonstrates complete and specific disappearance of the E2F4-p130-anti-E2F4 complex with the addition of the cyclin A antibodies. Some of the anti-cyclin A-containing complexes were retained in the well, which is characteristic of supershift with this specific antibody (40) and is not attributable to interaction with the free probe (Fig. 6C, rightmost lane). Not surprisingly, the cyclin A antibody did not affect the CDK2-deficient E2F4-p130-anti-E2F4 complexes of the DEX-treated postconfluent cells (Fig. 6D, right panel). As expected from the complete shift by the cyclin A antibody (Fig. 6C), cyclin E antibody did not affect any of the E2F complexes (data not shown). In summary, E2F4-p130-cyclin A-CDK2 becomes the predominant E2F-pocket complex in postconfluent MC3T3-E1 proliferating osteoblasts (Fig. 6C). Treatment of the postconfluent cells with DEX leads to elimination of cyclin A-CDK2 from this complex, resulting in a predominant stable E2F4-p130 complex (Fig. 6D), thus leading to attenuation of G1/S transition (Fig. 2).

GC Inhibit Cyclin A and CDK2 Expression Levels in Postconfluent MC3T3-E1 Cultures—DEX-induced elimination of cyclin A and CDK2 from E2F4-p130 complexes of postconfluent MC3T3-E1 cells (Fig. 6) could reflect either a decrease in the expression of these proteins or failure to associate with the E2F4-p130 moiety. To address this issue, Western analysis of nontreated and DEX-treated cells was performed. As demonstrated in Fig. 7A, DEX induced a ~5-fold decrease in the level of cyclin A (5.2 ± 2.6-fold in three independent experiments), and this was specific to the postconfluent stage (day 6). A
smaller or no reduction was observed in the abundance of D- and E-type cyclins (Fig. 7A). DEX also induced a significant decrease in the expression levels of CDK2 in the postconfluent cultures (2.6 ± 0.3-fold, n = 3) and in the level of CDK4 in both the pre- and postconfluent stages. Thus, the stage-specific effects of GC on MC3T3-E1 cell cycle progression (Fig. 2) and E2F-pocket complex composition (Fig. 6) best correlate with inhibition of cyclin A and CDK2 expression levels. These effects were also observed at the mRNA level (data not shown).

FIG. 5. Developmental and GC-induced alterations in E2F complexes in MC3T3-E1 osteoblasts. A, MC3T3-E1 cells were cultured in 100-mm plates and collected on either day 3 (preconfluency) or day 6 (postconfluency) 20 h following medium change, containing (DEX) or lacking (C) 1 μM DEX. Nuclear and cytoplasmic (Cyt) extracts were prepared, and gel shift assays were performed with an E2F probe as described under “Materials and Methods.” A, E2F4; ns, nonspecific complex. B and C, competition analyses of the E2F complexes of nontreated (B) or DEX-treated (C) extracts from preconfluent cells. The following reagents were added to the binding reaction: 100-fold excess of nonradiolabeled E2F oligonucleotide (wt; wild-type); 100-fold excess of nonradiolabeled E2F mutant oligonucleotide (mut; mutant); or antibodies to E2F, p107, or p130, as indicated. Antibodies to pRB did not induce any supershift or block shift (not shown). Arrow, p107; p130; arrowhead, unidentified E2F-pocket complex. Exposure times were adjusted to best demonstrate complexes of interest. Filled and open circles to the left of the band mark supershifted free E2F4 or supershifted E2F-pocket complex, respectively. D, Western blot analyses were performed as in Fig. 4B, using the same extracts as in A–C and antibodies against p130 or E2F4.

FIG. 6. Cyclin A-CDK2 distinguish E2F4-p130 complexes of nontreated compared with DEX-treated postconfluent MC3T3-E1 cells. Gel shift and competition assays were performed as in Fig. 5, with nuclear extracts from either nontreated (A, C) or DEX-treated (B, D) postconfluent (day 6) cells. In addition to E2F and pocket antibodies (A and B), cyclin-CDK2 antibodies were also employed here (C and D). Note that the E2F4-p130 complex of the nontreated cells (arrow), but not that of the DEX-treated cells (white arrow) contains cyclin A and CDK2. Ab in C indicates incubation of the probe with the cyclin A antibody in the absence of nuclear extracts.

FIG. 7. DEX decreases cyclin A and CDK2 levels in postconfluent MC3T3-E1 cultures. MC3T3-E1 cells growing to confluency (day 3) or to high density (day 6) were treated with either DEX (1 μM) or vehicle. The steroid was administered with the last medium change, 20 h prior to harvest. Protein extracts were prepared, and 60–80 μg was subjected to Western analysis as described under “Materials and Methods,” using anti-cyclin (A) or anti-CDK (B) antibodies.

**GC Decrease Cyclin A-, CDK2-, and Cyclin E-associated Kinase Activity in Postconfluent MC3T3-E1 Cells**—Persistence of inhibitory E2F-p130 complexes requires that CDK2 kinase activity be reduced (51). Immunoprecipitation and kinase assays were therefore performed with extracts of pre- and postconfluent MC3T3-E1 cells, either treated with DEX or untreated. As shown in Fig. 8A, the kinase activities associated with cyclin A and CDK2 were strongly reduced specifically in DEX-treated postconfluent cultures. In contrast, DEX only minimally decreased the kinase activity associated with cyclin D1 (Fig. 8A). Surprisingly, the kinase activity associated with postconfluent cyclin E was dramatically reduced by DEX (Fig. 8A) in the face of conserved protein levels (Fig. 7A). This led us to address the possibility that the reduced cyclin E-kinase activity could partly result from an increased association with CDK2 inhibitors. To this end, we exploited the heat resistance property of some CDK2 inhibitors, including p21 and p27, which allows estimation of their activity using a boiling/kinase inhibition assay (41, 42). Cyclin E immune complexes from nontreated or DEX-treated postconfluent MC3T3-E1 osteoblasts. A, MC3T3-E1 cells were cultured in 100-mm plates and collected on either day 3 (preconfluency) or day 6 (postconfluency) 20 h following medium change, containing (DEX) or lacking (C) 1 μM DEX. Nuclear and cytoplasmic (Cyt) extracts were prepared, and gel shift assays were performed with an E2F probe as described under “Materials and Methods.” A, E2F4; ns, nonspecific complex. B and C, competition analyses of the E2F complexes of nontreated (B) or DEX-treated (C) extracts from preconfluent cells. The following reagents were added to the binding reaction: 100-fold excess of nonradiolabeled E2F oligonucleotide (wt; wild-type); 100-fold excess of nonradiolabeled E2F mutant oligonucleotide (mut; mutant); or antibodies to E2F, p107, or p130, as indicated. Antibodies to pRB did not induce any supershift or block shift (not shown). Arrow, E2F4-p130; arrowhead, unidentified E2F-pocket complex. Exposure times were adjusted to best demonstrate complexes of interest. Filled and open circles to the left of the band mark supershifted free E2F4 or supershifted E2F-pocket complex, respectively. D, Western blot analyses were performed as in Fig. 4B, using the same extracts as in A–C and antibodies against p130 or E2F4.
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inhibition of both cyclin A- and cyclin E-associated CDK2 kinase activity and thus stabilization of inhibitory E2F-pocket complexes on promoters of cell cycle regulatory genes.

Osteoblasts That Have Grown to High Density Are Resistant to the Inhibitory Effect of GC on Differentiation—To begin addressing the significance of the developmental stage-specific antimitogenic effect of GC to bone formation, we initiated exposure of MC3T3-E1 cultures to DEX on day 3, 4, 5, or 6 (i.e. just before confluency, at confluency, and 1 or 2 days after confluency). The cultures were treated until day 21, and calcium deposition was evaluated histochemically and biochemically. The results demonstrate that DEX treatment commencing before confluency impedes the differentiation process, as reflected by >90% inhibition of calcium deposition (Fig. 9).

However, 1 day of DEX-free postconfluent condensation (i.e. commencement of DEX treatment on day 4) is sufficient to render the cultures partially resistant to GC exposure for the remaining of the culture period. When DEX treatment was initiated on day 6, after 3 days of DEX-free postconfluent condensation, calcium accumulation amounted to 64% of that measured in nontreated cultures (Fig. 9). Because calcium deposition in our MC3T3-E1 cultures starts around day 10–12, these data suggest that GC do not inhibit the mineralization process per se. Rather, a commitment step(s) associated with the postconfluent cell cycle is abrogated, leading to impaired differentiation.

Alleviation of GC Effects on Osteoblast Postconfluent Proliferation and Differentiation by the Antagonist/Partial Agonist RU486—The pleotropic effects of GC in cells may be explained, in part, by a multitude of mechanisms of action of the activated GR. These mechanisms include transcriptional activation and repression; autonomous action and interactions with other transcription factors (e.g. AP1, NF-kB); and DNA binding-dependent and -independent modes of action (reviewed in Ref. 53). Synthetic analogues, such as the partial agonist/antagonist RU486 have been employed to selectively mimic or block specific GC effects (54–56). The partial agonist/antagonist activity of RU486 in MC3T3-E1 cells is demonstrated in Fig. 10A using the MMTV promoter in transient transfection assays; some activation is observed with 0.1 μM RU486 alone (~6% of that observed with 0.1 μM DEX), and ~20-fold repression is observed when RU486 is added together with DEX. We rationalized that if GC inhibit osteoblast proliferation and differentiation via independent mechanisms, the two effects might be separable using RU486. However, RU486 alone did not mimic the effect of DEX on either osteoblast differentiation (Fig. 10B) or cell cycle progression (Fig. 10C). When administered together with DEX, RU486 blocked the inhibition exerted by DEX on both mineral deposition (Fig. 10B) and cell cycle progression (Fig. 10C). Thus, neither the partial agonist nor the antagonist activity of RU486 could dissociate the effects of
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The restoration of the cell cycle profile following DEX withdrawal prompted us to examine whether the inhibitory effect of DEX on both mineral deposition and postconfluent proliferation is reversible. As shown in Fig. 11A, MC3T3-E1 cultures were treated with 1 μM DEX starting on day 2 as in Fig. 2A. The last feeding, on day 10, either contained DEX as before (filled bars), or DEX was withdrawn (white bars). Cells were collected either 20 or 35 h after the last feeding, and cell cycle profiles were assessed as in Fig. 2 (mean ± S.D., n = 3). As shown in Fig. 11B, withdrawal of DEX resulted in restoration of the cell cycle profile following 10 days of treatment. Extracellular matrix mineralization was evaluated on day 15 by Alizarin red staining and quantitation of calcium accumulation as described under “Materials and Methods.”

Fig. 10. RU486 antagonizes the inhibitory effect of DEX on both mineral deposition and postconfluent proliferation. A, MC3T3-E1 cells were transiently transfected as described under “Materials and Methods” with a luciferase reporter gene driven by the MMTV promoter. On day 5, cultures were treated for 20 h with RU486 at the indicated concentrations, either without (top) or with 0.1 μM DEX (bottom), and harvested for luciferase assay (mean ± S.D., n = 3). B, MC3T3-E1 cells were cultured in 12-well culture dishes (3 × 10^4 cells plated per well) and treated in duplicate with either DEX, RU486, or both, at the indicated concentrations, starting on day 2. Left panel, mineralization of the extracellular matrix on day 10 is demonstrated by Alizarin red staining. Right panel, parallel wells were processed for quantitation of calcium deposition as described under “Materials and Methods” (mean ± S.D., n = 3). C, MC3T3-E1 cells were cultured as in Fig. 2B in the absence of steroids, and then treated acutely (20 h) with either 0.1 μM DEX (filled squares), 1 μM RU486 (open triangles), both drugs together (filled triangles), or vehicle (open circles). Cell cycle profiles were generated by flow cytometry as in Fig. 2. The percentage of cells in S + G2 + M is represented as mean ± S.D. of triplicate plates.

Fig. 11. The inhibitory effect of DEX on both mineral deposition and postconfluent proliferation is reversible. A, MC3T3-E1 cultures were treated with 1 μM DEX starting on day 2 as in Fig. 2A. The last feeding, on day 10, either contained DEX as before (filled bars), or DEX was withdrawn (white bars). Cells were collected either 20 or 35 h after the last feeding, and cell cycle profiles were assessed as in Fig. 2 (mean ± S.D., n = 3). B, MC3T3-E1 were cultured as in Fig. 10B and treated with 1 μM DEX during days 2–15 (DEX), during days 2–10 followed by 5 days without DEX (withdrawal), or they were not treated at all (Control). Extracellular matrix mineralization was evaluated on day 15 by Alizarin red staining (left) and quantitation of calcium accumulation (right; mean ± S.D., n = 3), as described under “Materials and Methods.”

suggest a linkage between the antimitogenic and antiphenotypic properties of glucocorticoids in osteoblasts.

DISCUSSION

Cell proliferation and differentiation are partially overlapping processes. During the differentiation course of MC3T3-E1 osteoblastic cells, a developmental switch occurs, which is responsible for persistent proliferation after confluency. Most notably, the cell cycle that drives growth to high density is inhibited by pharmacological doses of glucocorticoids, while the cell cycle driving growth toward confluency is not.

The cell cycle machinery of postconfluent MC3T3-E1 cultures is distinct from that operative in preconfluent cells. Some of the alterations in the cell cycle machinery, which occur during MC3T3-E1 growth to high density, typify cells undergoing growth arrest. Among these alterations are (i) an increase in p27 levels with a reciprocal decrease in p21 levels, as previously reported for rapamycin-arrested T lymphocytes (42) and contact-inhibited human fibroblasts (63); (ii) reduced free E2F DNA binding activity, as seen, for example, in growth-inhibited BALB/c 3T3 whole cell extracts (50) and in quiescent REF52 nuclear extracts (64); and (iii) preferential nuclear localization of E2F4, as in serum-starved NIH3T3 cells (65). The occurrence of these modifications in postconfluent MC3T3-E1 cultures suggests that the cell cycle persists in these cells despite some growth-inhibitory signal(s). Cooperativity be-
tween these signals and those elicited by GC may induce post-
confluent cell cycle inhibition.

MC3T3-E1 cells display a unique behavior of the RB-related protein p130. First, as opposed to many cell types in which p130 is a marker of growth arrest (51), cycling MC3T3-E1 cells constitutively express high p130 levels. Further, the protein is localized to the nucleus and found in E2F4-DNA complexes. In several previous studies demonstrating the unusual occurrence of E2F4-p130 complexes in cycling cells, these complexes have been shown to reside in the cytoplasm (38), or cellular localization has not been addressed (50, 66, 67). Significantly, the E2F4-p130 complexes of both pre- and postconfluent cycling MC3T3-E1 cells contain cyclin-CDK2; specifically at the post-
confluent stage, this complex is the main E2F-pocket complex.

It has been suggested that similar to E2F4-p107-cyclin-CDK2 complexes (52), the E2F4-p130-cyclin-CDK2 complexes repre-
sent an intermediate stage leading to release of free E2F4 (66). Free E2F4 binding activity would then promote G1/S transition (39).

Administration of DEX to postconfluent MC3T3-E1 cells re-
sults in attenuation of the G1/S transition. This antimitogenic effect is attributable to elimination of cyclin A and CDK2 from E2F4-p130 complexes, promoting the stability of the inhibitory E2F4-p130 complexes. Dissociation of CDK2 from E2F4-p107 (52) and E2F-p130 (66) complexes was suggested to be regulated by the CDI p21. However, the DEX-induced elimination of cyclin A-CDK2 from E2F4-p130 complexes at the postconfluent stage reflects down-regulation of their overall expression level, which also results in attenuation of the associated kinase activity. Although cyclin-CDK complexes are generally thought to be regulated by the cyclin, not the associated kinase activity. Therefore, it is possible that this cell cycle is an integral component of osteoblast differentiation, demonstrated here with our MC3T3-E1 subclone.

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Glucocorticoids Inhibit Developmental Stage-specific Osteoblast Cell Cycle: DISSOCIATION OF CYCLIN A-CYCLIN-DEPENDENT KINASE 2 FROM E2F4-p130 COMPLEXES
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