$p57^{Kip2}$, a Glucocorticoid-Induced Inhibitor of Cell Cycle Progression in HeLa Cells

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Glucocorticoids exert antiproliferative effects on a number of cell types, including the HeLa cervical carcinoma cell line. However, the mechanism responsible for the antiproliferative effect is poorly understood. In this report we have investigated the role of the recently identified cyclin-dependent kinase inhibitor (CDI) $p57^{Kip2}$ in the antiproliferative effect conferred by glucocorticoids. When HeLa cells were treated with the synthetic glucocorticoid dexamethasone (DEX), the doubling time of exponentially growing cells increased 2-fold. Within 11 h of DEX treatment, this was accompanied by an accumulation of cells in the G_1 phase of the cell cycle with a corresponding decreased proportion of cells in the S phase and decreased CDK2 activity. DEX treatment of the HeLa cells dramatically induced the protein and mRNA expression of the CDI $p57^{Kip2}$. This induction was seen within 4 h of DEX treatment, preceding a major DEX-induced accumulation of cells in the G_1 phase. DEX-induced mRNA expression of $p57^{Kip2}$ did not require *de novo* protein synthesis, and the transcription of the $p57^{Kip2}$ gene was increased as determined by a run-on transcription assay. Furthermore, DEX induction of $p57^{Kip2}$ was not a consequence of the cell cycle arrest, since other growth inhibition signals did not result in strong $p57^{Kip2}$ induction. Overexpression of $p57^{Kip2}$ using HeLa cells stably transfected with a tetracycline-inducible vector showed that $p57^{Kip2}$ is sufficient to reconstitute an antiproliferative effect similar to that seen in DEX-treated cells. Selective $p57^{Kip2}$ expression by the tetracycline analog doxycycline to levels comparable to those observed on DEX induction resulted in a 1.7-fold increase in the doubling time and a shift of HeLa cells to the G_1 phase as well as a decrease in CDK2 activity. Taken together, these results suggest that glucocorticoid treatment directly induces transcription of the $p57^{Kip2}$ gene and that the $p57^{Kip2}$ protein is involved in the glucocorticoid-induced antiproliferative effect. (Molecular Endocrinology 13: 1811–1822, 1999)

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INTRODUCTION

Glucocorticoids, produced in the adrenal cortex, are steroid hormones that play important roles in metabolism, immune responses, and cellular proliferation and differentiation (1). They bind to the intracellular glucocorticoid receptor (GR), a ligand-activated transcription factor. When liganded, GR interacts with specific DNA sequences, so-called glucocorticoid response elements (GREs) in target genes, increasing or decreasing their transcription. GR can also modify the activity of other transcription factors, *e.g*. AP-1 and nuclear factor-κB (NF-κB), through protein-protein interactions (reviewed in Ref. 2).

Glucocorticoids inhibit cell division in many tissues and cells, including those of lymphoid, fibroblastic, epithelial, and bone origin (1). Glucocorticoids have also been shown to inhibit proliferation of several cell lines, normally by causing an arrest in the G_1-phase of the cell cycle (3–7). However, the exact molecular mechanisms responsible for this effect remain unclear.

The propagation of the cell cycle is positively regulated by the action of a family of serine/threonine kinases called cyclin-dependent kinases (CDKs). Different CDKs are required in each phase of the cell cycle, and they are positively regulated after binding to regulatory subunits, cyclins, that are necessary for CDK activity. There are also several phosphorylation events that modulate the activity of CDKs (for review, see Ref. 8). Two families of proteins associate with specific CDK-cyclin complexes and inhibit their activity, thus acting as CDK inhibitors (CDIs). The first family, the Ink4 family, consists of four members (p15, p16, p18, and p19). These proteins specifically inhibit the activity of G_1 phase CDK4 or -6 cyclin D complexes, preventing entry into the S phase. The second family, the Cip/Kip family, has three members (p21, p27, and p57). To various extents, the proteins in this family bind to and inhibit CDK-cyclin complexes in all phases

of the cell cycle (reviewed in Ref. 9). $p21^{Cip1}$ and $p57^{Kip2}$ also inhibit DNA replication in a more direct way by binding to the proliferating cell nuclear antigen (10–13). $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ are highly expressed in differentiated cells and tissues (14–17). Despite this, mice lacking $p21^{Cip1}$ develop normally (18). This is also true for $p27^{Kip1}$-deficient mice, which develop essentially normally except for a general increase in body size, female sterility, and an increased frequency in pituitary tumorigenesis (19–21). $p57^{Kip2}$-deficient mice, on the other hand, display several severe and lethal phenotypes including changes in the gastrointestinal tract, shortened limbs, adrenal cortical hyperplasia, and abdominal muscle defects (22, 23). Interestingly, it has also been reported that $p57^{Kip2}$ expression is reduced in some human malignancies such as lung tumors and adrenocortical carcinomas (24, 25). $p57^{Kip2}$ is thus clearly important in normal development and may act as a tumor suppressor.

Inhibition of proliferation by several different families of hormones is mediated or correlated to an induction of CDIs. For instance, transforming growth factor-β (TGFβ) induces $p15^{Ink4B}$ and $p21^{Cip1}$ in human HaCaT keratinocyte cells (26, 27), and progesterone induces $p21^{Cip1}$ and $p27^{Kip1}$ in human T47D-YB breast cancer cells (28). Vitamin D_3 induces $p21^{Cip1}$ in human U937 monocyte-like cells (29) and $p27^{Kip1}$ in human HL60 leukemia cells (30). In human SMS-KCNR neuroblastoma cells, retinoic acid increases protein expression of $p27^{Kip1}$ (31), while interferon-α induces $p21^{Cip1}$ in Daudi and U-266 lymphoid cells and $p15^{Ink4B}$ in U-266 cells, respectively (32). Although little is known about the direct mechanism for the glucocorticoid-induced antiproliferative effects, it has been shown that glucocorticoids inhibit the expression of cyclin D3 and the protooncogene c-*myc* in P1798 murine T lymphoma cells (5, 33). This study also showed that overexpression of cyclin D3 and c-*myc* together was sufficient to overcome this antiproliferative block. Glucocorticoid-induced cell cycle block may also be due to an increased level of CDIs. In mouse L929 fibroblastic cells, rat BDS1 epithelial hepatoma cells, and rat lung alveolar epithelial cells, glucocorticoids induce the CDK inhibitor $p21^{Cip1}$ (7, 34–36). This regulation has been shown to involve both transcriptional and posttranscriptional mechanisms. In a study by Rogatsky *et al.* (4), the two human osteosarcoma cell lines, U2OS and SAOS2, were compared for mechanisms responsible for the antiproliferative effects of glucocorticoids. In SAOS2, a retinoblastoma protein (Rb)-deficient cell line, glucocorticoids increased the expression of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. However, in U2OS cells, which express Rb, glucocorticoids decreased the expression of cyclin D3, CDK4, and CDK6, without increasing the expression of CDIs. The results from this study suggest that the mechanism responsible for the antiproliferative phenotype induced by glucocorticoids may vary across different cell types. In most cases, a direct relation between the changes in CDI expression and the glucocorticoid-induced antiproliferative response is not clear.

It has previously been shown that glucocorticoids inhibit cellular proliferation in the human HeLa cervical carcinoma cell line either by causing an accumulation of cells in the G_1 phase (37, 38) or by prolongation of the G_2/M phase (39). In this study we have focused our investigation on the role of the Cip/Kip family of CDIs, particularly $p57^{Kip2}$, in the glucocorticoid-mediated inhibition of HeLa cell proliferation. The Cip/Kip family of CDIs has been implicated in inhibition of CDK-cyclin complexes not only in the G_1 phase of the cell cycle, but also in other cell cycle phases. Furthermore, the action of this family of CDIs is not dependent on Rb, which is absent in the HeLa cells (see below). We found that the synthetic glucocorticoid dexamethasone (DEX) inhibited proliferation of HeLa cells and that this antiproliferative effect correlated with a decrease in CDK2 activity and a major increase in both the mRNA and the protein expression of the CDI $p57^{Kip2}$. We also showed that $p57^{Kip2}$, on its own, was sufficient to partially reconstitute both the antiproliferative effect and the inhibition of CDK2 activity seen in glucocorticoid treated-cells.

RESULTS

DEX Induces an Accumulation of HeLa Cells in the G_1 Phase and Decreases the CDK2 Activity

After addition of the synthetic glucocorticoid DEX to HeLa cells, cell proliferation was inhibited (Fig. 1a). A 2-fold increase in the doubling time of an exponentially growing cell population after DEX treatment was observed compared with untreated cells, when a saturating concentration of DEX (100 nM) was used. The antiproliferative effect of DEX could be reversed by the glucocorticoid antagonist RU486, indicating that this is a GR-mediated effect (Fig. 1a). Administration of RU486 alone revealed no effect on the proliferation of the HeLa cells (data not shown).

To further characterize the DEX effect on cell cycle distribution in HeLa cells, the cells were subjected to flow cytometric analysis (Fig. 1b). As compared with untreated cells, 11 h of DEX treatment increased the number of cells in the G_1 phase from 62% to 75%, indicating a G_1 accumulation. This increase in the amount of cells in G_1 phase was accompanied by a decrease in the fraction of cells in S phase from 16% in untreated to 5% in treated HeLa cells. However, a complete growth arrest was not achieved as a significant number of cells remained in the S- and the G_2/M phases also after longer DEX treatment (data not shown). The changes in cell cycle distribution were visible after 11 h of DEX treatment, whereas after 5 h of treatment only a minor increase of the amount of cells in G_1 (61% in untreated as compared with 64% in treated) was observed. These results showed that DEX treatment induces an accumulation of cells in the G_1 phase within the first cell cycle after treatment, explaining the growth retardation seen in the proliferation assay.

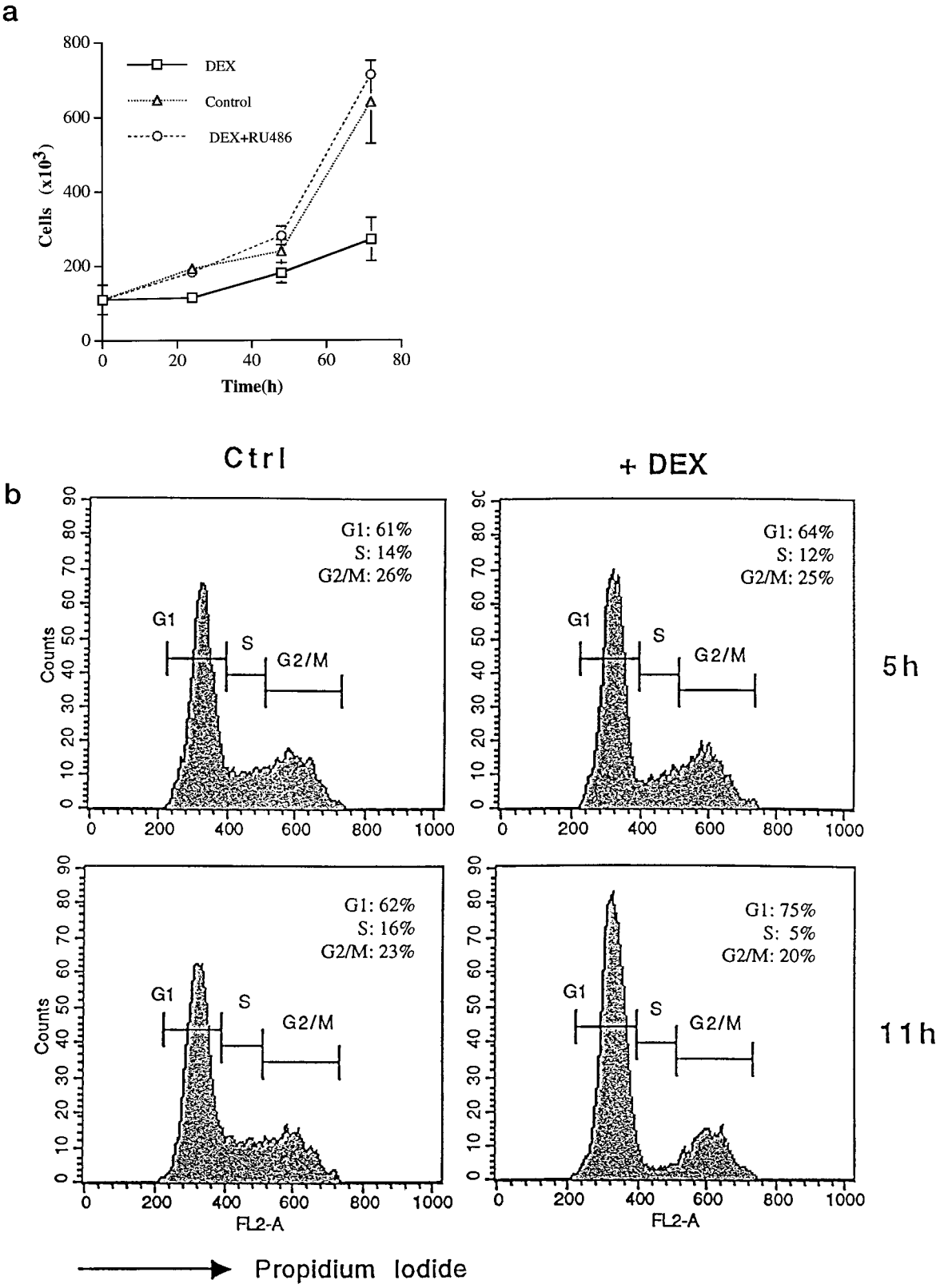

Fig. 1. The Effect of the Glucocorticoid Agonist DEX and the Antagonist RU486 on the Proliferation of HeLa tk⁻ Cells

a, Cells were treated with 100 nM DEX in the presence or absence of 1 μM RU486 and counted in a Bürker counter at 24-h intervals. Each measuring point is represented by triplicate samples from which the mean value and SD have been calculated. b, A representative flow cytometric analysis of asynchronously growing HeLa tk⁻ cells. The cells were cultured in the presence or absence of DEX (100 nM) for 5 h and 11 h, respectively.

The HeLa cells did not express any Rb as analyzed by Western blotting, while other human cell lines, *e.g.* HT29, gave a positive signal using the same assay conditions (results not shown). Since Rb is the key substrate for the CDK4/6-cyclinD complexes (40–42), we instead focused our investigation on the other positive regulator of G_1 progression, namely the CDK2-cyclin E complex. This was studied by immunoprecipitating CDK2 from HeLa whole-cell extracts and performing a Histone H1 kinase assay. The result showed that DEX treatment (100 nM) decreased the CDK2 activity (Fig. 2). This effect was already pronounced after 5 h and reached a maximal inhibition after 11 h, correlating well with the accumulation of cells in the G_1 phase seen after DEX treatment.

The Effect of DEX on Protein Expression of G_1-Acting Cell Cycle Proteins

As shown above, DEX treatment of HeLa cells caused a decrease in CDK2 activity. This glucocorticoid-mediated effect could either involve induction of different CDIs of the Cip/Kip family and/or down-regulation of the positively acting G_1 factors, CDK2 or cyclin E. We therefore analyzed the HeLa cells for expression of these proteins. Cells were treated with DEX for various time periods, after which they were harvested and whole-cell extracts (WCEs) prepared. The WCEs were subjected to immunoblot analysis, using antibodies against cyclin E, CDK2, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. As can be seen in Fig. 3, DEX dramatically induced the protein expression of $p57^{Kip2}$, whereas only minor inductions of the $p21^{Cip1}$ and $p27^{Kip1}$ proteins were observed. During the same time period the CDK2 level was not significantly changed, while the cyclin E expression was slightly decreased after 10 h and 24 h. The increase in $p57^{Kip2}$ level was seen as early as 4 h after treatment, and the maximal induction was observed after 8–10 h of treatment. This early induction of $p57^{Kip2}$ by DEX, which precedes any major G_1 accumulation (*cf*. Fig 1b where only a minor change in cell cycle distribution is seen after 5 h of DEX treatment), clearly suggests that the induction is a primary glucocorticoid-mediated effect and not secondary to a cell cycle block (see also below).

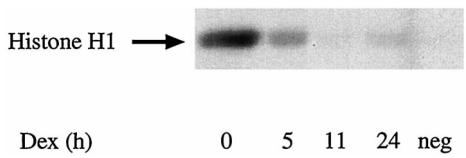

Fig. 2. CDK2 Activity in HeLa Cells after Glucocorticoid Treatment

CDK 2 activity as determined by Histone H1 phosphorylation in HeLa tk^- cells after DEX treatment (100 nM) for various time periods. As a negative control (neg.), an extract from untreated cells precipitated in the absence of the CDK 2 antibody was used.

A small induction of $p57^{Kip2}$ protein expression was detectable after treating HeLa cells with 1 nM of DEX for 24 h, while almost maximal induction was observed when the cells were exposed to 10 nM DEX (Fig. 4a). The responses seen with the different DEX concentrations are in line with the reported affinity of DEX for the GR [dissociation constant (K_d) ≈ 3 nM (43)]. Treating the cells with DEX together with a 10-fold excess of the antiglucocorticoid RU486 completely reversed the DEX-mediated induction of the $p57^{Kip2}$ expression (Fig. 4b). This also showed that RU486 acts as a pure

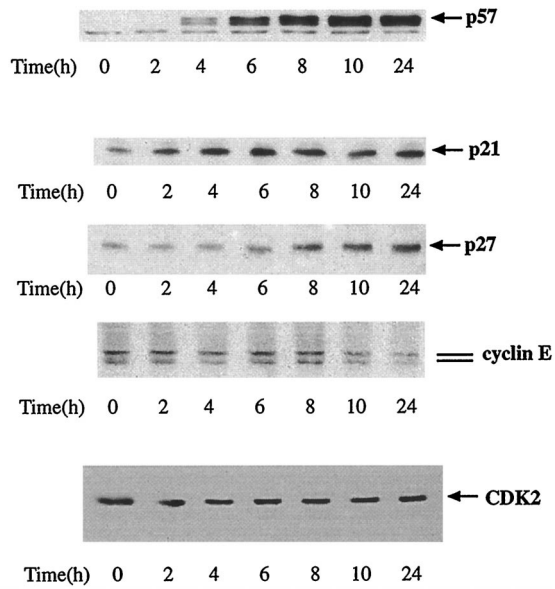

Fig. 3. $p57^{Kip2}$, $p21^{Cip1}$, $p27^{Kip1}$, CDK2, and Cyclin E Protein Expression in HeLa tk^- Cells after DEX Treatment

The cells were treated with DEX (100 nM) for various time periods and protein expression was analyzed by Western blotting, using antibodies directed to the different proteins.

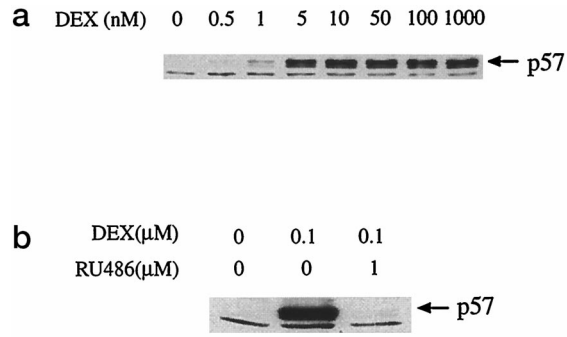

Fig. 4. Western Blot Analysis of $p57^{Kip2}$ Protein Expression in HeLa tk^- after Treatment with Different Concentrations of DEX

a, The cells were treated for 24 h with the DEX concentrations indicated in the figure. b, The effect of RU486 on the DEX-mediated induction of $p57^{Kip2}$ protein expression. The concentrations of DEX and RU486 used are indicated in the figure.

antagonist when it comes to p57Kip2 induction. The antagonistic effect of RU486 and the low concentration of DEX required to induce p57Kip2 protein expression further suggest that this is a GR-mediated effect.

The Effect of Alternative Growth-Inhibiting Signals on p57Kip2 Expression

To exclude the possibility that induction of p57Kip2 expression after DEX treatment is a secondary response to the cell cycle block, HeLa cells were exposed to alternative antiproliferative signals. Western blot analysis showed that serum starvation for 48 h only weakly induced p57Kip2 expression (Fig. 5a), while growing HeLa cells to confluency did not induce p57Kip2 expression (Fig. 5b). Under both conditions, DEX could induce p57Kip2 protein expression. HeLa cells were also blocked in late G_1/early S phase, using a double thymidine block. As a control for cell cycle arrest, [^{3}H]thymidine incorporation was measured, which showed that the cells retained only 3% of their DNA synthesis capacity after the block (result not shown). The double-thymidine block did not affect the p57Kip2 protein levels as determined by Western blotting (Fig. 5c). All these results further support the notion that the induction of p57Kip2 by DEX is a primary effect.

DEX Induces mRNA Expression as Well as Transcription of the p57Kip2 Gene

The short period of DEX treatment needed for induction of p57Kip2 protein expression (4 h, *cf*. Fig 3) suggested that the response is directly mediated by GR and does not require *de novo* protein synthesis. To test this hypothesis, we performed a Northern blot analysis on polyA-selected RNA from HeLa cells treated with DEX in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). The result, as shown in Fig. 6, revealed a single DEX-induced p57Kip2 transcript of ≈1.7 kb both in the presence and absence of CHX, comparable to the previously reported size for one of the p57Kip2 transcripts (16). As a control for RNA loading, the same membrane was hybridized with a probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 6, *lower panel*). Standardizing the p57Kip2 signal to the GAPDH signal showed that DEX induced p57Kip2 mRNA expression 14-fold in the absence of CHX and 9-fold in the presence of CHX. Under the conditions used, CHX blocked ≥ 85% of cellular protein synthesis as measured by [^{3}H]leucine incorporation (data not shown). This indicates that the majority of the DEX-mediated induction of p57Kip2 mRNA occurs in the absence of *de novo* protein synthesis, further supporting the notion of a direct GR-mediated effect.

The above results indicate that the p57Kip2 gene is transcriptionally regulated by GR. To examine this question, we performed a nuclear run-on transcription assay on nuclei prepared from HeLa cells treated with or without DEX for 6 h. The result showed that DEX strongly induced transcription of the p57Kip2 gene (Fig. 7), while transcription of the β-actin gene, which was used as a control for identical loading, was unaffected. No binding of radiolabeled RNA was seen to an empty vector (pcDNA3, result not shown).

p57Kip2 Expression on Its Own Is Sufficient to Induce Growth Inhibition in HeLa Cells

We next asked whether p57Kip2 on its own was sufficient to cause the antiproliferative phenotype seen in these cells. To investigate this, full-length human p57Kip2 cDNA was cloned into the tetracycline-induc-

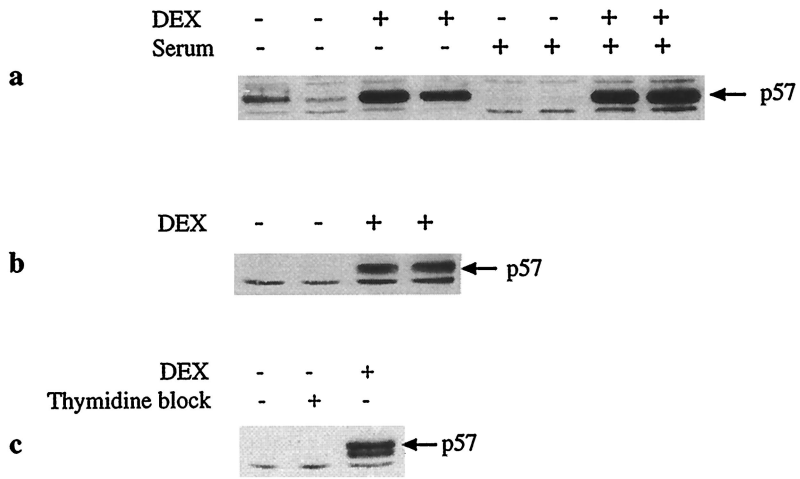

Fig. 5. The Effect of Serum Deprivation, Confluency, and Double-Thymidine Block on p57Kip2 Protein Expression in HeLa tk$^-$ Cells

a, The cells were cultured for 48 h in the presence or absence of serum and DEX (100 nM), as indicated in the figure. b, The cells were grown to confluency, after which they were cultured in the presence or absence of DEX (100 nM) for 24 h. c, The cells were subjected to a double thymidine block, and DEX (100 nM, 24 h)-treated cells were used as a positive control. p57Kip2 expression was analyzed by Western blot analysis using 20 μg of protein from WCEs.

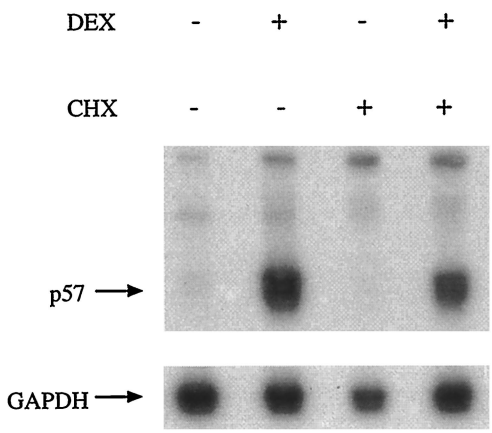

Fig. 6. The Effect of DEX Treatment on the Expression of the p57^{Kip2} mRNA

HeLa tk$^-$ cells were treated with or without DEX (1 μM) for 6 h in the presence or absence of CHX (5 μg/ml), after which p57^{Kip2} mRNA was detected by Northern blot analysis. The 1.7-kb p57^{Kip2} transcript is indicated in the figure (*upper panel*). As a control for RNA loading, the membrane was reprobed against GAPDH (*lower panel*). In the experiment 1 μg of poly A selected RNA was used in each lane. In the cases when CHX was used, it was added 1 h before DEX treatment.

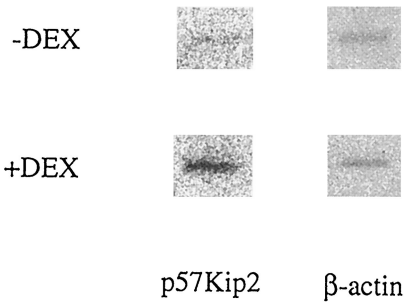

Fig. 7. p57^{Kip2} Transcription in HeLa tk$^-$ Cells Treated with or without DEX

A run-on transcription assay was performed on nuclei prepared from HeLa tk-cells nontreated or treated for 6 h with 100 nM DEX. An equal amount of ^{32}P-rUTP radiolabeled RNA (600 000 cpm/ml) from treated or nontreated cells was hybridized with a filter to which 3 μg of pcDNA3-hp57^{Kip2} or pGEM3-β-actin plasmid had been applied in each slot.

ible expression vector pTRE and stably transfected into HeLa Tet-On (CLONTECH Laboratories, Inc., Palo Alto, CA) cells. Several individual clones were isolated and screened for p57^{Kip2} transgene protein expression after stimulation with the tetracycline analog doxycycline (DOX). Examples of a p57^{Kip2} transgene-inducible (S9) and a noninducible (S8) clone are shown in Fig. 8. Notably, both clones induced endogenous p57^{Kip2} protein expression after DEX treatment. Furthermore, the magnitude of the DOX-mediated induction of transgene p57^{Kip2} expression in the S9 clone was similar to the DEX-mediated induction of endogenous p57^{Kip2} expression (Fig. 8).

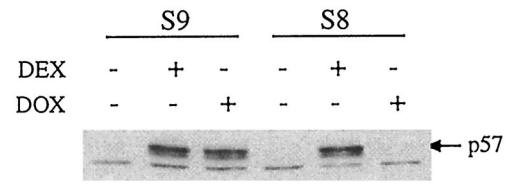

Fig. 8. Western Blot Analysis of p57^{Kip2} Expression in the S8 and the S9 Clones of HeLa Tet-On Cells Stably Transfected with the pTRE-p57^{Kip2} Tetracycline-Inducible Vector

The cells were treated for 15 h as indicated in the figure in the presence or absence of DEX (100 nM) or DOX (2 μg/ml), after which a Western blot analysis of p57^{Kip2} expression was performed.

The proliferation of the S8 and the S9 clones was investigated after DOX or DEX treatment. The doubling time of exponentially growing S9 cells increased 1.7-fold in the presence of DOX (Fig. 9a) as compared with untreated cells. In the control clone S8, the rate of proliferation after DOX treatment was essentially unaffected as compared with untreated cells (Fig. 9b). In contrast, DEX treatment increased the doubling time 2-fold in both clones.

Flow cytometric analysis of cell cycle distribution after DEX treatment of the S9 clone showed that the percentage of cells in the G_1 phase increased from 51% to 63% and was associated with a decrease in the amount of cells in G_2/M phase from 33% to 18% (Fig. 9c). Similarly, DOX treatment of the same clone increased the percentage of cells in G_1 phase from 51% to 56% and decreased the fraction of cells in G_2/M phase from 33% to 28%. Conversely, the S8 clone was unaffected by DOX treatment, while DEX treatment of S8 cells produced an increase of the amount of cells in G_1 phase from 48% to 61% (Fig. 9c). This shows that elevated p57^{Kip2} on its own is sufficient to induce antiproliferative effects and changes in cell cycle distribution resembling the ones observed in DEX-treated HeLa Tet-On cells. To mechanistically compare the DEX-induced and the DOX-induced antiproliferative effect in the S8 and S9 clones, we performed a CDK2 kinase assay on immunoprecipitated CDK2 from cells treated with or without DEX or DOX. The Histone H1 phosphorylation assay (Fig. 9d) showed that DEX inhibited the CDK2 activity both in the S8 and S9 clones, while DOX inhibited the CDK2 activity only in the transgene-inducible (S9) clone and not in the noninducible (S8) clone. Transgene induction of p57^{Kip2} thus inhibits CDK2 activity in HeLa cells, identically to the DEX-induced effect observed in these cells. In summary, all the results obtained from induction of the p57^{Kip2} transgene strongly support the involvement of this protein in the antiproliferative effect exerted by glucocorticoids on HeLa cells.

DISCUSSION

Glucocorticoids inhibit cell proliferation in a number of tissues and cell lines. This antiproliferative effect is

most often mediated by a cell cycle arrest in the G_1 phase (3–7). Different mechanisms have been reported for this arrest. In some model systems glucocorticoids decrease the levels of proteins that positively regulate cell cycle progression, *e.g*. c-*myc*, cyclin D3 (5, 33), and CDK4/6 (4). In other cells, glucocorticoids increase the levels of the CDIs p21^{Cip1} (4, 7, 34–36) and p27^{Kip1} (4). Thus, no consensus mechanism for this antiproliferative effect has been found and cell-specific mechanisms seem to operate.

We have investigated the role of the newly identified CDI p57^{Kip2} in the glucocorticoid-induced antiproliferative effect in HeLa cells. In our experiments, DEX induced accumulation of cells in the G_1 phase. This effect was clearly visible after 11 h of DEX treatment. Consistent with an earlier report (39), this was accompanied by a general enlargement of the cells together with a more flattened appearance (results not shown).

HeLa cells have been reported to express the human papilloma virus E7 protein (44), which sequesters and inactivates the Rb (for review see Ref. 45), one of the major tumor suppressors acting in the G_1 phase of the cell cycle. Furthermore, HeLa cells have been described to lack cyclin D-CDK4 complexes (46) and express little or no Rb (M. K. R. Samuelsson and S. Okret, result not shown). Since it is generally accepted that Rb is the key target for the CDK4/6-cyclinD-Ink4 signaling pathway, this pathway should not play a major role in the Rb-deficient HeLa cells (40–42, 47–49). An alternative mechanism for the glucocorticoid-induced G_1 effect is that glucocorticoids inhibit the activity of the second major G_1/S kinase activity, namely CDK2. CDK2 has been reported to phosphorylate p27^{Kip1}, triggering its degradation (50, 51), and may also modify other proteins that are involved in origin firing and DNA replication (52, 53). These mechanisms may be alternative pathways by which CDK2 operates in Rb-deficient cells. Indeed, we found that DEX treatment of HeLa cells decreased the activity of CDK2 and that the time needed for this inhibition is consistent with the time needed for induction of the G_1 accumulation. Thus, we focused our investigation on whether the change in CDK2 activity could be explained by glucocorticoid-mediated alterations in expression of CDK2, cyclin E, and/or the Cip/Kip family of CDIs. The Cip/Kip family of inhibitors interacts with and inhibits most CDK-cyclin complexes. These proteins can, at least theoretically, inhibit cell cycle progression by acting in all cell cycle phases, although overexpression of the Cip/Kip family of CDIs in different cell lines primarily causes a G_1 arrest (for review see Ref. 9).

Our results showed that DEX treatment of HeLa cells dramatically induced p57^{Kip2} protein expression and reduced CDK2 activity within a few hours. During the same time period the protein levels of CDK2 and cyclin E were essentially unchanged, while the p21^{Cip1} and p27^{Kip1} levels were only marginally elevated. To our knowledge, this is the first demonstration of an external signal directly regulating p57^{Kip2} expression, although a recent publication (54) showed ubiquitin-dependent proteosomal degradation of p57^{Kip2} after TGFβ1 treatment of primary rat osteoblastic cells. The induction of p57^{Kip2} protein was detectable after 4 h of DEX treatment and thus it preceded the major G_1 accumulation seen in these cells. The induction occurs pretranslationally, since DEX also induces p57^{Kip2} mRNA expression. This induction does not require *de novo* protein synthesis, and we were able to show, using a run-on transcription assay, that the p57^{Kip2} gene is transcriptionally induced by glucocorticoid treatment. Furthermore, no DEX-mediated change in the protein stability of the endogenous p57^{Kip2} was found, since its rate of degradation was identical to the rate of degradation of the transgene p57^{Kip2} induced by DOX in the tetracyclin-inducible S9 clone (result not shown). In summary, these experiments suggest that DEX-mediated induction of p57^{Kip2} expression is a primary effect caused directly by DEX and not a secondary effect caused by the cell cycle inhibition itself. This is further supported by the observation that other antiproliferative signals such as serum deprivation, confluence, and a double-thymidine block do not induce p57^{Kip2} protein expression at all or only to a very small extent.

By stably transfecting HeLa cells with the human p57^{Kip2} cDNA under the control of a tetracycline (DOX)-inducible promoter, we were able to show that p57^{Kip2} alone could induce an antiproliferative effect similar to that observed after DEX treatment of the cells. Furthermore, induction of p57^{Kip2} with either DEX or DOX induced an accumulation of cells in the G_1 phase as well as a decrease in the CDK2 activity, supporting an important role for p57^{Kip2} in this process. Thus, we were able to show a functional interaction between p57^{Kip2} and CDK2 in HeLa cells, correlating well with earlier studies demonstrating a direct protein-protein interaction between these proteins (16, 17). Despite similar phenotype induced by p57^{Kip2} alone and DEX treatment, we cannot exclude the involvement of additional factors in the antiproliferative effect exerted by glucocorticoids on HeLa cells; for instance, cyclin E expression is slightly decreased, and both p21^{Cip1} and p27^{Kip1} protein levels are slightly increased after DEX treatment. Furthermore, the morphological changes of the HeLa cells observed after DEX treatment were not seen when p57^{Kip2} was expressed on its own, suggesting another mechanism for this effect (M. K. R. Samuelsson, result not shown). Interestingly, a recent paper by Kaltschmidt *et al.* (55) showed that IκB-α overexpression in HeLa cells led to cell cycle inhibition as well as morphological changes similar to those observed in the DEX-treated cells. This opens up the possibility that the full antiproliferative response induced by glucocorticoids in HeLa cells is a combination of transcriptional induction of the p57^{Kip2} gene and transrepression of NF-κB by GR (2). The reason why DEX treatment of the HeLa Tet-On cells results in a smaller DEX effect on the S phase as compared with the HeLa tk$^-$ cells is unclear, but could

be due to the fact that the cells (and clones), which originate from different sources, are not completely identical. This is in line with reports from various groups describing slightly different effects of DEX on the cell cycle phases in HeLa cells (37–39).

Although we have shown that $p57^{Kip2}$ on its own is sufficient to induce an antiproliferative effect in HeLa cells mimicking the effect after DEX treatment, it may not be necessary for this effect. We have attempted to investigate this issue by performing several experiments aiming at reducing the DEX-mediated induction of $p57^{Kip2}$. Techniques such as transfections with antisense constructs, antisense phosphorthioate DNA oligonucleotides, and stable transfections with hammerhead ribozymes have been used. In none of these experiments were we able to efficiently down-regulate the DEX-mediated induction of the $p57^{Kip2}$ protein expression (results not shown). There could be several reasons for this, *i.e.* the induction of $p57^{Kip2}$ is too powerful to be efficiently counteracted by any antisense expression or degradation by ribozymes or the high GC content of the $p57^{Kip2}$ transcript makes it unavailable for the antisense constructs (56).

As mentioned above, DEX increased the level of $p57^{Kip2}$ mRNA in HeLa cells, without any requirement for *de novo* protein synthesis. We also showed that the $p57^{Kip2}$ gene is transcriptionally regulated by glucocorticoids. Several putative GREs have been identified within the human $p57^{Kip2}$ promoter when a computer search was performed (data not shown). It is posssible that GR exerts its effect by binding to one or several of these elements. Alternatively, GR may function through binding to other transcription factors. The latter mechanism has been shown for progesterone receptor activation of the $p21^{Cip1}$ promoter, which occurs through a Sp1 site (57).

Further investigations must be performed to test whether the induction of $p57^{Kip2}$ by glucocorticoids is a general mechanism. We have screened several cell lines known to be growth inhibited by glucocorticoids and found $p57^{Kip2}$ induction after DEX treatment in some, but not all, of them (result not shown). This shows that the induction of $p57^{Kip2}$ does not only occur in HeLa cells, suggesting that this regulation may be a common mechanism in the antiproliferative action of glucocorticoids.

In summary, we report a novel mechanism for glucocorticoid-mediated inhibition of cell proliferation, involving transcriptional induction of the $p57^{Kip2}$ gene. $p57^{Kip2}$ has earlier been shown to be important for embryonic development in mice (22, 23) and has been proposed to be a tumor suppressor in humans (24, 25). In connection with this, it is interesting to note that glucocorticoid treatment can inhibit tumor promotion as in the case of the mouse skin carcinoma model (58, 59). Further experiments will reveal a possible connection between glucocorticoids and $p57^{Kip2}$ in these processes.

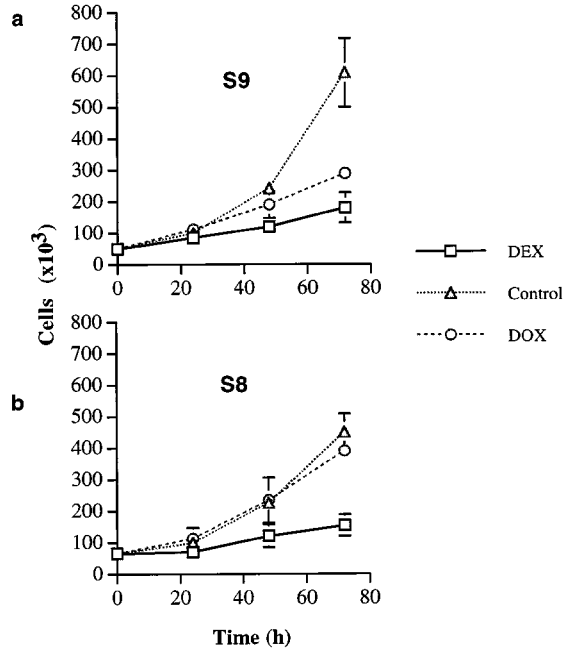

Fig. 9. The Effect of DEX and DOX on Proliferation, Cell Cycle Distribution, and CDK2 Activity of HeLa Tet-On Cells Stably Transfected with the pTRE-$p57^{Kip2}$ Tetracycline-Inducible Vector

a, The $p57^{Kip2}$ transgene-inducible clone S9 was subjected to DEX (100 nM) or DOX (2 μg/ml) treatment for various time periods. b, The noninducible control clone S8 was subjected to DEX (100 nM) or DOX (2 μg/ml) treatment for various time periods. In both cases the amount of cells were counted in a Bürker chamber. Each measuring point is represented by triplicate samples from which the mean value and SD have been calculated. c, Flow cytometric analysis was performed on asynchronously growing cells from the S8 and the S9 clone, after treatment with DEX (100 nM) or DOX (2 μg/ml) for 15 h as indicated in the figure. Shown here is one representative analysis from each clone. d, CDK2 kinase activity as determined by Histone H1 phosphorylation in the $p57^{Kip2}$ transgene inducible clone S9 and the noninducible clone S8 after treatment with DEX (100 nM) or DOX (2 μg/ml) for 15 h. As a negative control (neg.) an extract from untreated S9 cells precipitated without the CDK2 antibody was used.

MATERIALS AND METHODS

Cell Culture Conditions and Hormone Treatment

The two strains of human cervical carcinoma HeLa cells used in this study, HeLa tk^- (ATCC, Manassas, VA) and HeLa Tet-On (CLONTECH Laboratories, Inc.), were cultured in MEM supplemented with MEM nonessential amino acids (1×), sodium pyruvate (1 mM), penicillin-streptomycin (100 IU/ml), L-glutamine (2 mM) and FBS (10%). All cell culture medium was obtained from Life Technologies, Inc. (Paisley, UK). All cells were grown at 37 C in a humidified atmosphere of 90% H_2O and 5% CO_2.

DEX (Sigma Chemical Co., St. Louis, MO) and RU486 (Roussel-UCLAF, Romanville, France) were dissolved in 99.5% ethanol. In all experiments where DEX or RU486 was added to the cells, the same final concentration of ethanol was used as a control for solvent effects.

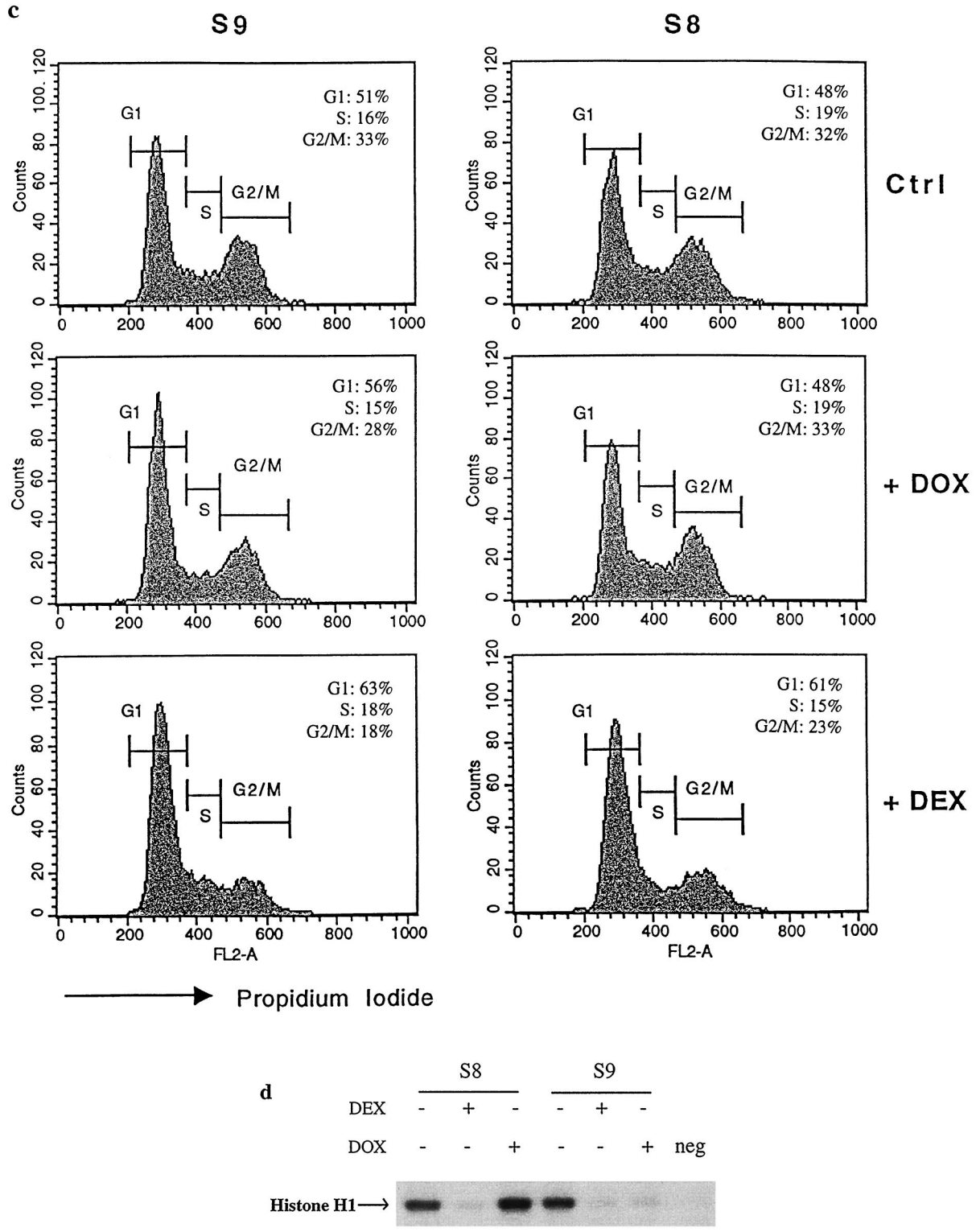

Fig. 9. Continued.

Proliferation Assay

For cell proliferation assays, cells were seeded in six-well multidishes (Nunc, Roskilde, Denmark). After hormone treatment, cells were harvested at 24-h intervals by treatment with Trypsin-EDTA (Life Technologies, Inc.), single-cell suspensions were prepared, and cells were counted in a Bürker chamber. Triplicates from each time point were analyzed. The doubling time for each condition was calculated from cells in exponential growth phase.

Flow Cytometric Analysis

For flow cytometric analysis 1–2 × 10^6 cells were washed with sample buffer (PBS supplemented with 1 g/liter glucose), fixed in cold (−20 C) 70% ethanol and stored at −20 C overnight. The samples were centrifuged and the pellets were resuspended in sample buffer containing propidium iodide (50 μg/ml) (Sigma Chemical Co.) and DNase-free RNase A (50 μg/ml) (Roche Molecular Biochemicals, Mannheim, Germany) for 1 h at room temperature while the tubes were vigorously agitated on a shaking platform. Ten thousand cells were analyzed by flow cytometry in a FACSscan (Becton Dickinson and Co., Franklin Lakes, NJ). Living cells were gated to exclude debris and clumps.

Immunoprecipitation and Histone H1 Kinase Assay

HeLa cell extracts for immunoprecipitation were prepared by sonication in the absence of any detergent, and the subsequent immunoprecipitation and Histone H1 kinase assays were performed according to the protocol described by Sangfelt *et al*. (32). For each reaction, 50 μg protein were immunoprecipitated with a polyclonal rabbit antihuman CDK2 antibody (15536E, PharMingen, San Diego, CA) (1:1000). Extracts treated without the antihuman CDK 2 antibody were used as negative controls.

Western Blot Analysis

WCEs for Western blot analysis were prepared by lysing the cells in ice-cold Nonidet P-40 (NP-40) buffer (0.5% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA) for 10 min, after which cell debris was removed by centrifugation (14,000 × *g*, 10 min, 4 C). An equal volume of 2× SDS gel-loading buffer (60) was then added and the samples were boiled for 2 min. Protein concentrations in cell extracts were quantitated spectrophotometrically before addition of the loading buffer with the Bio-Rad protein assay kit, according to the instructions of the manufacturer (Bio-Rad Laboratories, Inc., Hercules, CA). Twenty micrograms of protein from each whole cell extract were electrophoretically separated on a 9% SDS-polyacrylamide gel and electroblotted onto a Hybond C-extra membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). To check for equal loading and transfer, the membranes were stained with Ponceau red (Sigma Chemical Co.). For protein detection, the immunoblots were then probed either with a rabbit IgG polyclonal anti-$p57^{Kip2}$ antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:100), a rabbit IgG polyclonal anti-$p21^{Cip1}$ antibody (C-19, Santa Cruz Biotechnology, Inc.) (1:100), a mouse IgG monoclonal anti-$p27^{Kip1}$ antibody (Transduction Laboratories, Lexington, KY) (1:2000), a rabbit polyclonal IgG anticyclin E antibody (M-20, Santa Cruz Biotechnology, Inc.) (1:50), and a rabbit polyclonal IgG anti-CDK2 antibody (M2, Santa Cruz Biotechnology, Inc.) (1:50). As secondary antibodies, either a horseradish peroxidase-conjugated antirabbit or an antimouse IgG antibody (Amersham Pharmacia Biotech) (1:3000) was used. The membranes were then subjected to enhanced chemiluminescence (Amersham Pharmacia Biotech, UK) and autoradiography, according to the instructions of the manufacturer.

Double Thymidine Block of HeLa Cells

HeLa cells were cultured as described before and subsequently subjected to a double thymidine block as described by Stein *et al.* (61). According to the same protocol, [^{3}H]thymidine incorporation was measured as a control for cell cycle arrest. This showed that the cells only retained less than 3% of their DNA synthesis capacity after the block (result not shown).

Northern Blot Analysis

HeLa cells were incubated in the presence or absence of DEX (1 μM) and/or CHX (5 μg/ml) (Sigma Chemical Co.) for 6 h. When CHX was used, it was added 1 h before the addition of DEX. Poly A selection of cellular RNA was carried out using the Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway) according to the instructions of the manufacturer. Northern blot analysis was carried out according to Ref. 62, using 1 μg of poly A selected RNA. The 0.24–9.5 kb RNA ladder from Life Technologies, Inc. was used as a size marker. The Northern blot filters (Hybond-N+, Amersham Pharmacia Biotech) were hybridized with a ^{32}P-labeled cRNA probe, first against $p57^{Kip2}$ and subsequently against GAPDH. All probes were synthesized by using the Riboprobe *in vitro* transcription kit (Promega Corp., Madison, WI), according to the instructions of the manufacturer. As a template for the $p57^{Kip2}$ riboprobe, we used a pBSKSII+ plasmid (Promega Corp.) with the human $p57^{Kip2}$ cDNA cloned into it (kindly provided by Dr. S. J. Elledge, Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, TX) and as a template for the GAPDH riboprobe we used a linearized antisense probe template (Ambion, Inc. Austin, TX). Quantification of the hybridization signals were made using a BasIII phosphoimager (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Run-on Transcription Assay

Preparation of cell nuclei from HeLa cells treated with or without DEX for 6 h was performed as described in Current Protocols in Molecular Biology (62a). The subsequent run-on transcription assay detecting $p57^{Kip2}$ and β-actin transcripts was performed as follows: 50 μl transcription buffer (50 mM HEPES, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 μg/ml BSA, 300 mM NH_4Cl), 5 μl rATP, 5 μl rGTP, 5 μl rCTP (all 10 mM, Promega Corp.), 1 μl Rnasin (Promega Corp.), 2 μl Heparin (5 mg/ml), 10 μl ^{32}P-rUTP (10 μCi/μl, Amersham Pharmacia Biotech), and 50 μl nuclei were incubated for 20 min at 26 C. Ten units of DNase (RNase free, Promega Corp.) were added and incubated for 30 min at 37 C, after which 400 μl Proteinase K buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 2 mM KCl, 1 mM EDTA, 0.5% SDS] and 10 μl proteinase K (10 mg/ml) were added and incubated for 30 min at 37 C. After adding 12 μl 4 M NaCl and 16 μl tRNA (16 mg/ml), a phenol-chloroform extraction was performed. The radiolabeled RNA was subsequently precipitated once with trichloroacetic acid (10%) and once with LiCl (1 M) + ice-cold ethanol (50%). Each filter was hybridized (Hybond-N+, Amersham Pharmacia Biotech) for 72 h at 45 C with 600,000 cpm/ml of radiolabeled RNA as determined with a scintillation counter. The filters were washed with 0.2×SSC, 0.1% SDS at 72 C for detection of the $p57^{Kip2}$ transcript, and at 60 C for the β-actin transcript, and analyzed by autoradiography. The reason for the different washing temperatures used was that the $p57^{Kip2}$ transcript hybridized more strongly than the β-actin transcript due to a high GC content in the $p57^{Kip2}$ cDNA (56). The plasmids used as probes were the pcDNA3-$hp57^{Kip2}$ (the human $p57^{Kip2}$ cDNA cloned into the *Eco*RI site of the pcDNA3 vector (Invitrogen, Carlsbad, CA)) and the pGEM3-β-actin vector (63). The plasmids were linearized and denatured, and 3 μg were added to each slot and blotted on to the filter by a slot blot apparatus (Src 072/0 Minifold II, Schleicher & Schuell, Inc., Dassel, Germany).

Transfection Conditions and Selection of Stably Transfected Cells

The human $p57^{Kip2}$ cDNA was subcloned into the *Eco*RI site of the tetracycline-inducible pTRE vector and transfected together with the pTK-Hyg selection vector into HeLa Tet-On cells using the Lipofectin transfection reagent (Life Technologies, Inc.) according to the instructions of the manufacturer.

A 5:1 molar ratio of pTRE-hp57Kip2 to pTK-Hyg was used. Selection was performed in 200 μg/ml of Hygromycin B (Life Technologies, Inc.). After selection, individual clones were isolated and continuously cultured in the presence of Hygromycin B. To induce transgene p57Kip2 expression, the cells were treated with 2 μg/ml of doxycycline hydrochloride (Sigma Chemical Co.).

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