CDK9 Is Constitutively Expressed throughout the Cell Cycle, and Its Steady-State Expression Is Independent of SKP2

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CDK9 is a CDC2-related kinase and the catalytic subunit of the positive-transcription elongation factor b and the Tat-activating kinase. It has recently been reported that CDK9 is a short-lived protein whose levels are regulated during the cell cycle by the SCF^{SKP2} ubiquitin ligase complex (R. E. Kiernan et al., Mol. Cell. Biol. 21:7956-7970, 2001). The results presented here are in contrast to those observations. CDK9 protein levels remained unchanged in human cells entering and progressing through the cell cycle from G_0, despite dramatic changes in SKP2 expression. CDK9 levels also remained unchanged in cells exiting from mitosis and progressing through the next cell cycle. Similarly, the levels of CDK9 protein did not change as cells exited the cell cycle and differentiated along various lineages. In keeping with these observations, the kinase activity associated with CDK9 was found to not be regulated during the cell cycle. We have also found that endogenous CDK9 is a very stable protein with a half-life ($t_{1/2}$) of 4 to 7 h, depending on the cell type. In contrast, when CDK9 is overexpressed, it is not stabilized and is rapidly degraded, with a $t_{1/2}$ of less than 1 h, depending on the level of expression. Treatment of cells with proteasome inhibitors blocked the degradation of short-lived proteins, such as p27, but did not affect the expression of endogenous CDK9. Ectopic overexpression of SKP2 led to reduction of p27 protein levels but had no effect on the expression of endogenous CDK9. Finally, downregulation of endogenous SKP2 gene expression by interfering RNA had no effect on CDK9 protein levels, whereas p27 protein levels increased dramatically. Therefore, the SCF^{SKP2} ubiquitin ligase does not regulate CDK9 expression in a cell cycle-dependent manner.

CDK9 is the catalytic subunit of the positive-transcription elongation factor b (P-TEFb) (30). CDK9 was first identified as a CDC2-related kinase named PITALRE, which associated with a number of cellular polypeptides and exhibited a kinase activity that was not regulated during the cell cycle (14). CDK9 associates with either cyclin T1, T2a, T2b, or K to form active P-TEFb complexes, which activate transcriptional elongation by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (RNAPII) as well as negative-transcription elongation factors (reviewed in reference 26). CDK9 is tethered to promoters by certain transcription factors, such as NF-κB, the androgen receptor, and c-myc, resulting in stimulation of transcription (2, 8, 18). CDK9/cyclin T1 complexes are also the target of the human immunodeficiency virus transcriptional activator Tat, which recruits this complex to the *trans*activation response structure in the nascent human immunodeficiency virus RNA and positions the kinase complex to phosphorylate the CTD of the RNAPII, and other transcription factors, resulting in stimulation of transcriptional elongation that would otherwise be interrupted (26).

CDK9 has also been found to associate with components of a chaperone pathway, including HSP70, HSP90, and CDC37

(23). CDK9 seems to form separate complexes with HSP70, the HSP90/CDC37 complex, and cyclin T1. However, the cyclin T1/CDK9 complex is the only mature complex with kinase activity (23). It has been proposed that the chaperone pathway recognizes newly synthesized CDK9 and helps in the proper folding of the protein, which ultimately associates with cyclin T1 (23).

It has recently been reported that CDK9 is a short-lived protein whose levels are regulated during the cell cycle by changes in protein stability resulting from the cell cycle-dependent modulation of SKP2 (17). SKP2 is an F-box protein component of the SCF^{SKP2} ubiquitin ligase, which acts as a receptor for substrates that are then degraded by the proteasome (16). SKP2 has been shown to target p27 and p130 for degradation and is, at least in part, responsible for the modulation of p27 and p130 protein levels during the cell cycle (4, 6, 9, 15, 22, 27–29). It has also been proposed that cyclin T1 targets CDK9 for degradation (17).

Because neither the expression of CDK9 (J. Garriga and X. Grana, unpublished data) nor the expression of its regulatory subunit cyclin T1 (11) is modulated during the cell cycle and because changes have not been seen in the activity of CDK9 in cells progressing synchronously through the cell cycle upon release from a hydroxyurea G_1/S block (14), we reexamined whether SKP2 modulates CDK9 expression during the cell cycle, as was recently proposed, and the potential involvement of cyclin T1 in this process (17).

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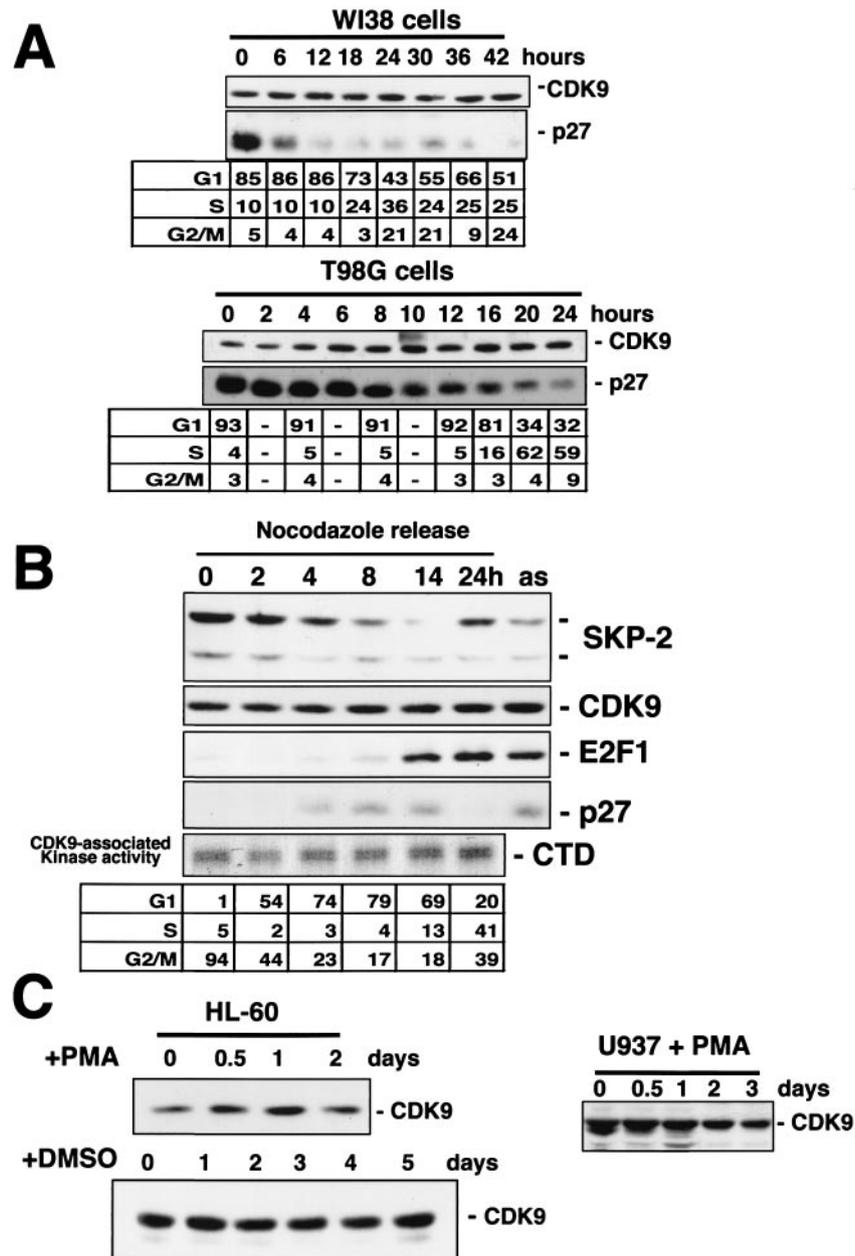
FIG. 1. CDK9 protein expression and kinase activity are not cell cycle regulated. (A) CDK9 protein levels in cells entering and progressing through the cell cycle from G_0. WI38 primary human fibroblasts (upper panels) and T98G human glioblastoma cells (lower panels) were serum starved and restimulated as described in Materials and Methods, and cells were collected at the indicated time points. CDK9 and p27 protein levels were analyzed by Western blotting with anti-CDK9 and anti-p27 antibodies, respectively. The percentage of cells at each phase of the cell cycle was determined by flow cytometric analysis and is indicated in the tabular portions of the panels. (B) CDK9 protein expression and kinase activity in cells progressing through the cell cycle after mitotic arrest. HeLa cells were incubated in the presence of nocodazole for 16 h. Mitotic cells resumed the cell cycle after being placed in fresh, nocodazole-free medium. The cells were collected at the indicated time points. CDK9, SKP2, E2F1, and p27 protein levels were analyzed by Western blotting by using their respective antibodies. Kinase assays were performed for each time point after protein extracts were immunoprecipitated with anti-CDK9 antibodies. GST-RNAPII-CTD was used as the exogenous substrate (see Materials and Methods). as, asynchronous. (C) CDK9 protein expression does not change during myeloid cell differentiation. Exponentially growing HL60 and U937 cells were induced to differentiate to macrophages in the presence of PMA (3 ng/ml). HL-60 cells were also induced to differentiate to granulocytes in the presence of 1.3% DMSO. Cells were collected at the indicated time points, and the expression levels of CDK9 protein were assessed by Western blot analysis.

MATERIALS AND METHODS

Cell culture. HeLa, T98G, 293, WI38, and H1299 cells were grown in Dulbecco modified Eagle medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Sigma). HL60 and U937 cells were grown in RPMI medium (Cellgro) plus 10% heat-inactivated FBS. Exponentially growing HL60 and U937 cells (0.2 × 10^6 to 0.4 × 10^6 cells/ml) were induced to differentiate to macrophages by supplementation of the medium with 3 ng of phorbol 12-myristate 13-acetate (PMA; Sigma) per ml. Exponentially growing HL60 cells were induced to differentiate to granulocytes in the presence of 1.3% dimethyl sulfoxide (DMSO). T98G and WI38 cell growth was synchronized by serum deprivation
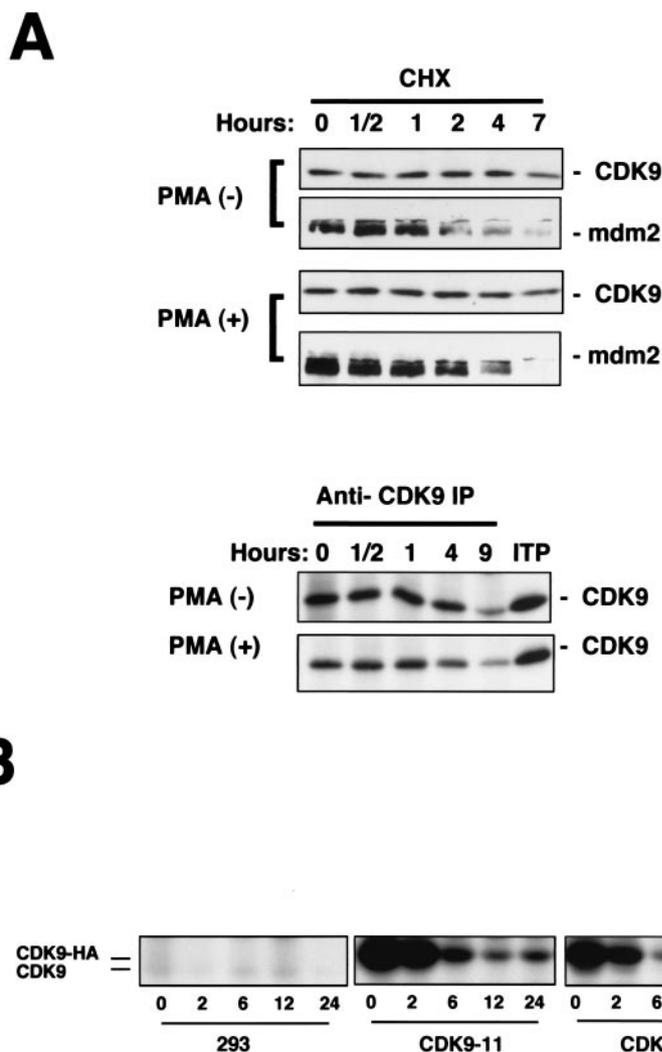
FIG. 2. Endogenous CDK9 is not a short-lived protein. (A) CDK9 protein $t_{1/2}$ was analyzed in exponentially growing HL60 cells (−PMA) or HL60 cells differentiating to macrophages (+PMA) by pulse-chase and CHX treatments. HL60 cells were treated with CHX (40 µg /ml) to block protein synthesis for the indicated time periods. CDK9 and mdm2 protein levels were analyzed by Western blotting by using anti-CDK9 and anti-mdm2 antibodies (upper panels). HL60 cells were labeled with [^{35}S]Met and analyzed by pulse-chase. CDK9 was immunoprecipitated by using anti-CDK9 antibodies (lower panels). ITP, in vitro-translated protein. (B) The $t_{1/2}$s of endogenous and ectopically expressed CDK9 proteins were analyzed by pulse-chase labeling (see above and Materials and Methods) in 293 cells and in two 293-derived cell lines (CDK9-11 and CDK9-14) that constitutively express CDK9-HA (ectopically expressed CDK9 carrying an HA tag).

and made to reenter the cell cycle after restimulation with serum (20, 21). HeLa cell growth was synchronized by treatment with nocodazole (50 ng /ml) (21). Flow cytometric analysis was performed essentially as described previously (20) with a FACScan (Becton Dickinson).

Antibodies. Anti-CDK9 (sc-484), anti-cyclin T1 (sc-10750), anti-SKP2 (sc-7164), anti-SKP1 (sc-7163), anti-p27 (sc-528), and anti-E2F1 (sc-193) rabbit polyclonal antibodies and antihemagglutinin (anti-HA; sc-7392) mouse monoclonal antibodies were obtained from Santa Cruz Biotechnology. Anti-HA (12 CA5) mouse monoclonal antibodies were obtained from Roche, anti-Cul1 (C32620) mouse monoclonal antibodies were obtained from BD Transduction Laboratories, and anti-myc (OP10F) mouse monoclonal antibodies were obtained from Oncogene Research Products.

Protein assays. Whole protein extracts were prepared by lysing cells in a solution containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na$_3$VO$_4$, 2 mM phenylmethylsulfonyl fluoride, 10 µg of leupeptin/ml, 4 µg of aprotinin/ml, and 4 µg of pepstatin/ml (lysis buffer). Immunoprecipitation and Western blotting were performed as previously described (20). Briefly, whole protein extracts or immunoprecipitated complexes were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Protogel; National Diagnostics), transferred to poly-

vinylidene difluoride membranes (Immobilon-P; Millipore) in 10 mM 3-(cyclohexylamino)propanesulfonic acid (pH 11) containing 10% methanol, and detected by using horseradish peroxidase-conjugated secondary antibody (Amersham) and Enhanced Chemiluminescence reagent (Dupont). Kinase assays were performed as described previously(10, 12, 14). Briefly, kinase assays were performed with anti-CDK9 immunoprecipitates at 30°C for 20 min in 20 mM HEPES (pH 7.4)–10 mM magnesium acetate–1 mM dithiothreitol–20 µM ATP (2 × 10^4 to 4 × 10^4 cpm/pmol) in the absence or presence of exogenous substrates (glutathione *S*-transferase [GST]-RNAPII-CTD) in a total volume of 35 µl. The reaction was terminated by the addition of 35 µl of 2× Laemmli sample buffer. GST-RNAPII-CTD was obtained as previously described (11, 13).

Protein stability assays. (i) Pulse-chase experiments. Cells were incubated for 30 min in methionine- and cysteine-free DMEM (GIBCO) supplemented with 10% dialyzed FBS (GIBCO). Expres^{35}S protein labeling mix (NEN Life Science Products) was added to this medium (0.1 mCi/ml), and the cells were incubated for another 2 h. After the 2-h incubation, the cells were washed with phosphate-buffered saline (PBS) and incubated in regular DMEM–10% FBS (293 cells) or RPMI medium–10% heat-inactivated FBS (HL60 cells) for the time periods indicated in the figures. After the chase, the cells were lysed and anti-CDK9 immunoprecipitations were performed as described above. In vitro-translated

CDK9 was generated by using the TNT reticulocyte system (Promega) and pBS-CDK9 as a template.

(ii) CHX treatment. Protein synthesis was inhibited in various cell lines by adding cycloheximide (CHX) to the medium (40 μg/ml) for the time periods indicated in the figures. Cells were collected at the time points indicated in the figures, and whole protein lysates were processed for Western blot analysis by using anti-CDK9 antibodies (see above).

(iii) Lactacystin treatment. Protein degradation by the proteasome system was inhibited in serum-restimulated T98G cells by adding lactacystin (10 μM) to the medium for 5 h. After the 5-h incubation, cells were collected at the time points indicated in the figures, and whole protein lysates were processed for Western blot analysis (see above).

Transfections. Cells from the 293 cell line were plated at a density of 60 to 80% in 100-mm-diameter plates and grown overnight. The cells were refed 4 h before transfection. Transfections were carried out by the standard calcium phosphate DNA precipitation procedure (1, 10). The cells were harvested 48 h after transfection. (See the figure legends for the specific DNA used in each experiment.) Stably transfected CDK9-expressing 293 cell lines were described earlier (10). pCMV-CDK9-HA (10), pCDNA3-HA-cyclin T1 (25), pCDNA3-SKP2, pCDNA3-SKP1, and pCDNA3-Cul1 (27) have been described previously.

Ad generation. Adenoviruses (Ads) were generated as reported earlier (3). Recombinant Ads expressing cyclin T1 or CDK9 were generated by homologous recombination of plasmid pJM17, which contains the Ad genome except regions E1 and E3, and a shuttle vector, pΔE1sp1A, in which we subcloned the cDNA of interest under the control of a cytomegalovirus (CMV) promoter. The recombination event occurred in 293 cells after they were cotransfected with the two plasmids. Recombinant Ads can replicate in this cell line only because it provides the E1 proteins in *trans*. When recombination occurs, the resulting Ad completes the life cycle, lysing the host cells. Expression of the recombinant protein was tested in the cell lysate. Next, single Ad clones were isolated by infecting 293 cells and overlaying the monolayer with agarose and DMEM. Individual viral plaques were picked and pure Ad clones were eluted from the agarose and subsequently amplified, infecting 293 cells. The recombinant Ads were purified with CsCl from lysates obtained from 293 cells, and the titer was assessed by plaque formation.

Ad infection. T98G cells were infected by adding CsCl-purified Ad to the serum-free medium at a multiplicity of infection (MOI) of 100 PFU/cell, and the cells were harvested at the time points indicated in the figures. HeLa cells were infected at different MOIs as indicated below. CsCl-purified Ads were added to the medium (DMEM plus 2% FBS) for 3 h, after which the cells were replenished with fresh medium and harvested after 48 h. The control Ad, which expresses β-galactosidase (Ad–β-gal), was used to normalize the number of PFU per cell.

RNAi. We used the mammalian expression vector pSUPER to synthesize SKP2-short interfering RNA (siRNA)-like transcripts (5). The constructs were made by following the authors' directions (available at www.nki.nl/nkidep/agami /downloads/manualpsuper.doc). The sequences targeted by the siRNA that we chose are in the N terminus (5′-GAGGAGCCCGACAGTGAGA-3′) and in the C terminus (5′-GTTGGAGCTGGATTGCT-3′) of SKP2. Since these short sequences are susceptible to point mutations, we tested several plasmids derived from each sequence and chose the most effective (see Fig. 6). Cells from the 293 and H1299 cell lines were transiently cotransfected by using the calcium phosphate precipitation method with 1 μg of pCDNA3-SKP2 and increasing amounts (1 to 20 μg) of different pSUPER-RNA interference (RNAi)-SKP2 clones of each of the two constructs. The effectiveness of each pSUPER-RNAi-SKP2 plasmid was assessed by monitoring downregulation of ectopically expressed SKP2 2 days posttransfection by Western blot analysis. Next, the same cell lines were cotransfected with 10 μg of pSUPER-RNAi-SKP2 and 1 μg of pSG5puro. After 1 week of selection with puromycin (1 μg/ml), pools of resistant cells were collected and whole protein lysates were processed for Western blot analysis to assess changes in the levels of expression of relevant cellular proteins.

RESULTS AND DISCUSSION

It has previously been shown that CDK9 activity is not modulated in HeLa cells progressing synchronously throughout the cell cycle upon release from a hydroxyurea arrest at the G_1/S transition (14). A recent report has suggested that CDK9 levels are regulated in a cell cycle-dependent manner by the SCF^{SKP2} ubiquitin ligase complex (17). Kiernan et al. showed that the expression of SKP2 correlates inversely with the expression of the CDK9 protein. Obviously, such dramatic regulation of
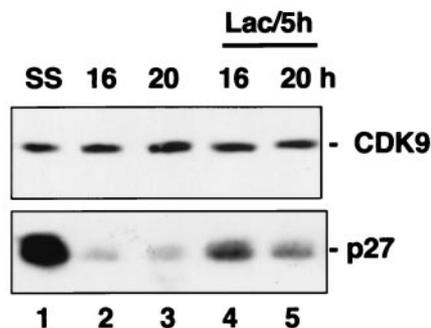
FIG. 3. Lactacystin does not affect the expression of endogenous CDK9. T98G cells were serum starved and restimulated as described in Materials and Methods. The cells were collected 16 and 20 h after restimulation. One set of samples was treated with lactacystin for 5 h before the cells were collected (11 and 15 h after restimulation). The levels of CDK9 and p27 proteins in extracts of cells treated with lactacystin or vehicle were determined by Western blot analysis using anti-CDK9 and anti-p27 antibodies, respectively. Lac/5h, treatment with lactacystin for 5 h; SS, serum starved.

CDK9 expression would be predicted to have an effect on CDK9-associated kinase activity. In view of these contrasting findings, we have determined the levels of expression of CDK9 protein throughout the cell cycle in a variety of primary and tumor cells.

Human WI38 primary lung fibroblasts and T98G glioblastoma cells were serum starved for 3 days and then restimulated with fresh medium supplemented with 10% FBS as previously described (20). The cells were harvested at the indicated time points and processed for Western blotting and flow cytometric analysis following propidium iodide staining. Figure 1A shows that while the levels of p27 are dramatically downregulated following serum stimulation of serum-starved cells, CDK9 expression is constitutive. p27 expression is known to be regulated primarily at the protein level by changes in protein stability that are, at least in part, dependent on SKP2 (15, 16, 19).

Next we determined whether the levels of CDK9 were regulated during exit from mitosis and progression through G_1 upon release from a transient mitotic block. HeLa cells were incubated with nocodazole for 16 h. Loosely attached cells, which are arrested at mitotic metaphase, were obtained by gentle shaking of the culture dishes, collected, washed twice with PBS, and reseeded in fresh complete medium, essentially as described previously (21). The cells were harvested at the indicated time points and processed for Western blotting and flow cytometric analysis. Figure 1B clearly shows that, unlike those of p27 and E2F1, CDK9 levels are not regulated throughout the cell cycle. Similar results were obtained with T98G cells treated under the same conditions (data not shown). We also found that CDK9-associated kinase activity was not modulated during mitosis exit and entry nor during progression through the next cell cycle (Fig. 1B, lower panel). We next determined whether changes in the levels of CDK9 protein expression could be detected when cells are induced to exit the cell cycle and differentiate. Figure 1C shows that CDK9 expression is not regulated during PMA-induced differentiation of HL-60 and U937 cells through the monocyte/macrophage lineage or DMSO-induced differentiation of HL-60 cells to granulocytes. We have also seen lack of regulation of CDK9 expression
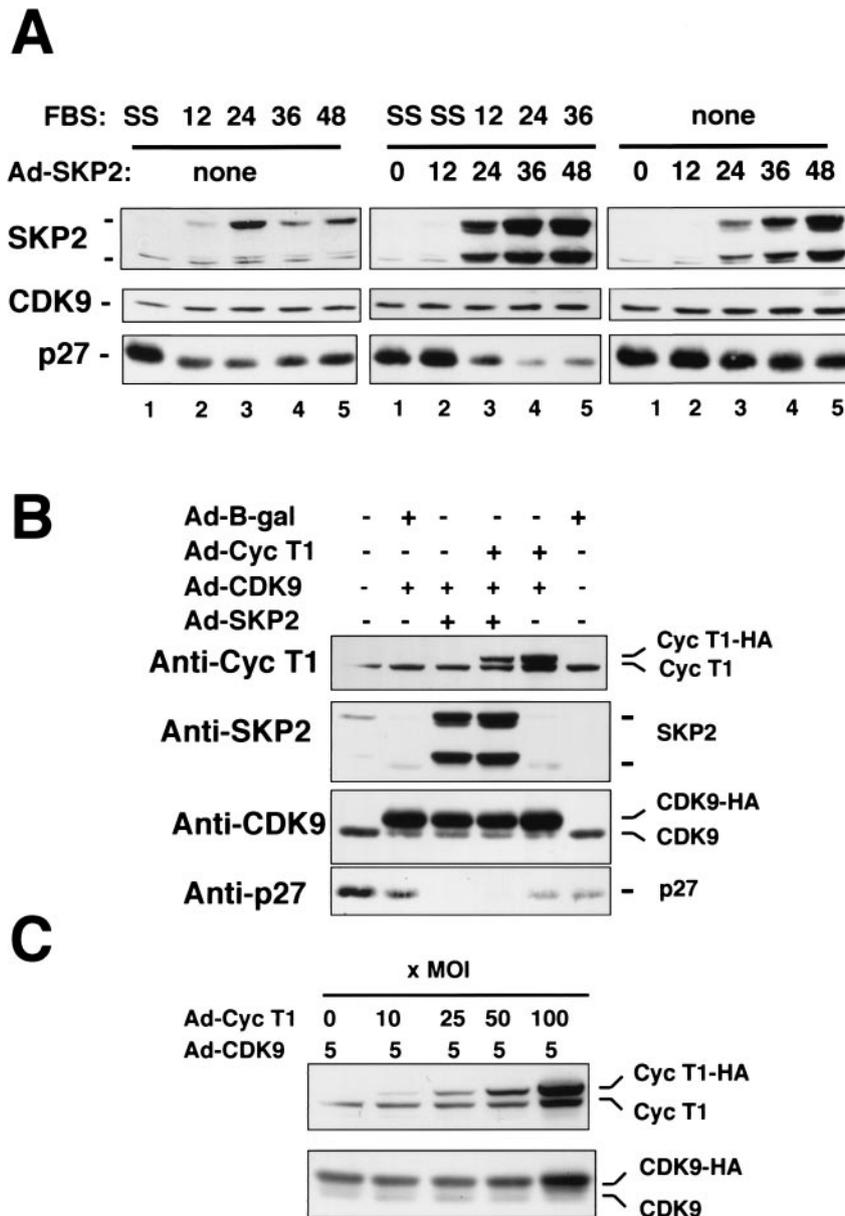
FIG. 4. Ectopic expression of SKP2 does not affect the expression of CDK9. (A) T98G cells were serum starved and restimulated (left panels); serum starved, infected with recombinant Ad expressing SKP2 (Ad-SKP2), and restimulated (middle panels); or serum starved and infected with Ad-SKP2 (right panels). Cells were collected at the indicated time points after restimulation or infection, and the expression levels of SKP2, CDK9, and p27 proteins were analyzed by Western blotting. SS, serum starved. (B) Exponentially growing HeLa cells were infected with the indicated recombinant Ad. The cells were collected 48 h after infection, and the expression levels of SKP2, CDK9, and p27 proteins were analyzed by Western blotting. (C) Effect of the ectopic expression of cyclin T1 on CDK9 stability. HeLa cells were infected with combinations of recombinant Ad expressing CDK9-HA (Ad-CDK9; constant MOI) and recombinant Ad expressing cyclin T1-HA (Ad-cyclin T1; increasing MOI). Forty-eight h postinfection, the cells were collected and the expression levels of cyclin T1 and CDK9 proteins were analyzed by Western blotting in the cell lysates.

during skeletal muscle, erythroid, and neuronal cell differentiation (Garriga et al., unpublished data). Therefore, we conclude that the expression of the CDK9 protein is not regulated in a cell cycle-dependent manner.

The SCF^{SKP2} ubiquitin ligase complex is involved in targeting short-lived proteins for degradation by the proteasome (reviewed in reference 16). Thus, we determined the stability of the CDK9 protein in a variety of cell types by measuring the kinetics of CDK9 degradation. The half-life ($t_{1/2}$) of the CDK9

protein in proliferating and differentiating HL-60 cells was determined by performing CHX and [^{35}S]methionine-cysteine pulse-chase analysis. Treatment of HL60 cells with CHX, an inhibitor of protein synthesis, for the indicated periods of time showed that CDK9 is a stable protein. The $t_{1/2}$ of CDK9 was found to be more than 4 h, while the levels of mdm2 (24), a short-lived protein, decreased rapidly (Fig. 2A, upper panels). A metabolic pulse with [^{35}S]methionine-cysteine followed by a chase for the indicated periods of time exhibited similar results
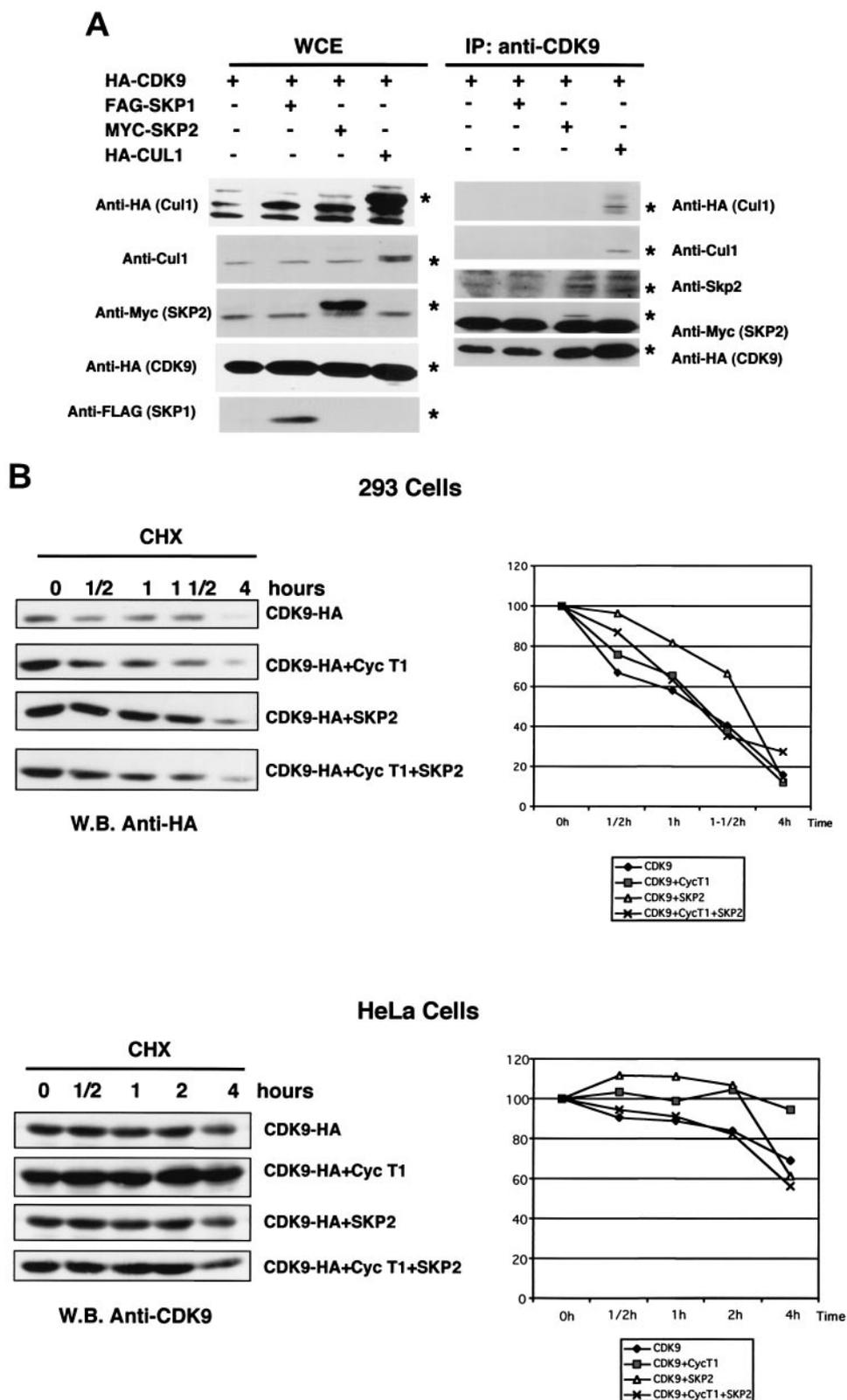
FIG. 5. CDK9 interacts with SKP2 and Cul-1 in transfected 293 cells. (A) Cells from the 293 cell line were transfected with pCMV-CDK9-HA (HA-CDK9), pCMV-Flag-SKP1 (FAG-SKP1), pCMV-myc-SKP2 (MYC-SKP2), and pCMV-HA-Cul1 (HA-Cul1) in the combinations indicated. Five micrograms of each plasmid was transfected together with 0.5 μg of a luciferase reporter plasmid by the calcium phosphate method. The cells were collected 48 h following transfection, and whole-cell extracts were immunoprecipitated with anti-CDK9 antibodies. Whole-cell extracts and immunoprecipitates were resolved by SDS-PAGE followed by Western blot analysis. The left panels show the expression levels of the transfected proteins, determined by using specific antibodies. The right panels show coimmunoprecipitation of Cul-1 and SKP2 with CDK9. SKP1 could not

(Fig. 2A, lower panels). Likewise, the $t_{1/2}$ of the CDK9 protein in U2-OS and T98G cells was also found to be more than 4 h (data not shown). Other studies have noticed that when CDK9 is ectopically overexpressed, it becomes unstable (10, 23; Garriga et al., unpublished data). Figure 2B shows a comparison of the $t_{1/2}$s of endogenous and exogenous CDK9 in 293 cells determined by using [^{35}S]methionine-cysteine pulse-chase assays. When CDK9 is overexpressed in 293 cells, it is rapidly degraded, with a $t_{1/2}$ of less than 2 h (Fig. 2B, middle and right panels). In contrast, when CDK9 is expressed at its physiologic levels (endogenous CDK9 in 293 cells), it is more stable, with a $t_{1/2}$ of more than 6 h (Fig. 2B, left panel). Similar results were obtained with CHX assays (see Fig. 5B). These results are consistent with previous observations that CDK9 is stabilized by a chaperone pathway that involves HSP70 and HSP90/CDC37 before CDK9 forms a very stable complex with cyclin T1 and the suggestion that unprotected monomeric CDK9 is rapidly degraded (23). These results are, however, in contrast to results reported recently (17) suggesting that endogenous and exogenous CDK9 are both short-lived proteins.

Because of the suggestion that the SCF^{SKP2} ubiquitin ligase complex could mediate CDK9 degradation through the proteasome pathway (17), we decided to determine whether lactacystin, an inhibitor of the proteasome, would have any effect on CDK9 expression during the cell cycle. T98G cells were serum starved for 3 days and then restimulated with serum. Two sets of plates were collected at 16 and 20 h following serum stimulation. One set of plates was treated with lactacystin for the last 5 h of the time course, while the other set was treated with vehicle. As expected and shown in Fig. 3, p27 downregulation throughout the cell cycle was prevented by inhibition of the proteasome (compare lanes 2 and 3 with lanes 4 and 5). However, CDK9 expression did not change throughout the G_0/G_1 transition, and its expression was not affected by inhibition of the proteasome. Similarly, treatment of exponentially growing HL-60 cells with lactacystin had no effect on CDK9 expression (data not shown).

To further investigate the potential role of SKP2 in targeting CDK9 for degradation, we ectopically expressed SKP2 by using recombinant Ads in serum-starved T98G cells and exponentially growing HeLa cells. Stimulation of serum-starved T98G cells with 10% FBS led to upregulation of endogenous SKP2 and downregulation of p27 (Fig. 4A). As shown in Fig. 1A and 3, CDK9 levels remained unchanged. Ad-mediated expression of SKP2 in serum-starved T98G cells led to robust expression of exogenous SKP2. However, expression of SKP2 in the absence of serum was not sufficient to induce downregulation of p27 during the indicated time course (Fig. 4A, right panels). This is likely due to the fact that phosphorylation of p27 precedes its recognition as a substrate by the SCF^{SKP2} ubiquitin

ligase complex. This phosphorylation is induced by signaling that is dependent on mitotic stimulation. Ectopic expression of SKP2 followed by restimulation with 10% FBS led to a more potent downregulation of p27 in cells than treatment with FBS alone (compare p27 expression levels at 24 and 36 h following serum stimulation [Fig. 4A, left and middle panels]). In contrast, CDK9 levels were not affected by the ectopic expression of SKP2 in either the presence or the absence of serum. To determine whether ectopic expression of SKP2 affects the expression of endogenous and exogenous CDK9 in exponentially growing cells, we infected HeLa cells with Ad-SKP2, Ad-cyclin T1, or the Ad–β-gal control with or without Ad-CDK9 and determined the expression levels of SKP2, p27, CDK9, and cyclin T1 48 h later. Ectopic expression of CDK9 resulted in downregulation of endogenous CDK9, as reported earlier (10, 23). Expression of SKP2 resulted in downregulation of endogenous p27 but did not affect the expression of either endogenous or exogenous CDK9, even when cyclin T1 was overexpressed (Fig. 4B). Thus, dramatic changes in the expression of SKP2, which led to changes in the expression of p27, one of its known substrates, did not alter CDK9 expression in vivo. We also determined whether expression of cyclin T1 in HeLa cells had any effect on the expression of both endogenous and exogenous CDK9. HeLa cells were transduced with Ad-CDK9 and increasing amounts of Ad-cyclin T1, and the expression levels of CDK9 and cyclin T1 were determined by Western blot analysis. Figure 4C shows that the expression of cyclin T1 does not reduce the steady-state expression of endogenous CDK9. In contrast, when highly overexpressed, cyclin T1 stabilizes exogenous CDK9. These results contradict the model proposed recently (17), in which cyclin T1 would target CDK9 for degradation by the proteasome via its interaction with the SCF^{SKP2} ubiquitin ligase complex.

Given that we could not find any relationship between the expression levels of the SKP2 and CDK9 proteins, we decided to reexamine whether CDK9 and the components of the SCF^{SKP2} ubiquitin ligase complex interact in vivo when transfected in cells as reported recently (17). Interestingly, we could coimmunoprecipitate SKP2 and Cul-1 with CDK9 by using specific antibodies (Fig. 5A). SKP1 was not detected in the complex, most likely because of the presence of comigrating bands corresponding to the light chain of the primary antibody, which are recognized by the secondary antibody used in the Western blot analysis (data not shown). We conclude that, at least when overexpressed in transfection experiments, CDK9 interacts with a SCF^{SKP2} ubiquitin ligase complex.

Given that overexpression of CDK9 dramatically decreases the $t_{1/2}$ of the CDK9 protein (Fig. 2B) (23), we asked whether coexpression of cyclin T1, as suggested earlier (17), and/or SKP2 could alter CDK9 protein stability. CDK9-, SKP2-, and

be detected in this experiment (see text for details). Asterisks indicate the migration of specific bands. WCE, whole-cell extracts; IP, immunoprecipitates. (B) Ectopic expression of SKP2 and cyclin T1 alone or in combination does not affect CDK9 $t_{1/2}$. CDK9-, SKP2-, and cyclin T1-expressing plasmids were transfected into 293 cells alone or in combination, as indicated in the upper panels. In parallel experiments, HeLa cells were transduced with Ads expressing CDK9, SKP2, and cyclin T1 alone or in combination (lower panels). Twenty-four hours posttransfection and posttransduction, the cells were divided onto five identical plates. Twenty-four hours later, the cells were incubated with CHX (40 µg/ml) for the indicated periods of time. The expression levels of CDK9 at each time point were analyzed by Western blotting. In each case, Western blots from a representative experiment as well as quantification of the results of three independent experiments are shown. Expression levels are represented as percentages of the expression values at time zero. W.B., Western blot.
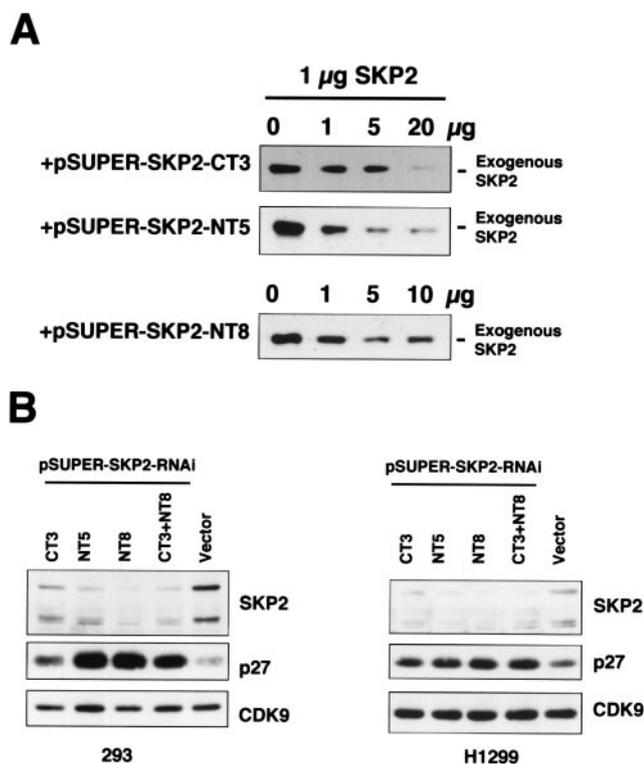
A

B

FIG. 6. Downregulation of cellular SKP2 gene expression by RNAi has no effect on CDK9 protein levels. The mammalian expression vector pSUPER was used to synthesize SKP2-RNAi-like transcripts. (A) The effectiveness of each of the pSUPER-RNAi-SKP2 constructs was determined in transient-transfection experiments. H1299 cells were transiently cotransfected with 1 μg of pCDNA3-SKP2 and increasing amounts of the indicated pSUPER-RNAi-SKP2 constructs. Forty-eight hours after transfection, the cells were collected and whole protein extracts were resolved by SDS–10% PAGE followed by Western blot analysis using anti-SKP2 antibodies. (B) Cells from the 293 cell line and H1299 cells were cotransfected with 10 μg of different pSU-PER-RNAi-SKP2 constructs and 1 μg of pSG5puro. After 1 week of selection with puromycin, pools of resistant cells were collected and whole protein extracts were resolved by SDS–12% PAGE followed by Western blot analysis using the indicated antibodies. The expression levels of cellular SKP2, p27, and CDK9 are shown.

cyclin T1-expressing plasmids were transfected into 293 cells alone or in combination, as indicated in Fig. 5B (upper panels). Similarly, HeLa cells were transduced with Ads expressing CDK9, SKP2, and cyclin T1, as indicated in Fig. 5B (lower panels). Twenty-four hours posttransfection and posttransduction, the cells were divided onto five identical plates. Twenty-four hours later, the cells were incubated with CHX for the periods of time indicated in the figure. Figure 5B shows that the $t_{1/2}$ of ectopically expressed CDK9 is not significantly reduced by coexpression of SKP2, cyclin T1, or both. Graphs represent the average of the results of three independent experiments (see legend to Fig. 5).

To directly determine whether SKP2 endogenous levels are rate limiting for the regulation of CDK9 expression, we downregulated endogenous SKP2 in human cells by using siRNA. We used the mammalian expression vector pSUPER (5) to synthesize SKP2-RNAi-like transcripts. Figure 6A shows the effectiveness of various pSUPER-SKP2 constructs in down-

regulating exogenous SKP2 in transient-transfection experiments. Next, we used these same constructs along with a puromycin resistance plasmid to transfect both 293 and H1299 cells. After selecting the transfected cells with puromycin for a week, we collected populations of the resistant cells and analyzed the expression levels of endogenous SKP2, CDK9, and p27 (Fig. 6B). Expression of SKP2 was effectively downregulated by the SKP2-RNAi-like transcripts. As expected, this led to the upregulation of p27. However, CDK9 levels remained unchanged.

In summary, our experiments clearly show that endogenous CDK9 protein expression is not regulated during the cell cycle despite major changes in the expression of SKP2, which inversely correlates with the expression of p27, a known substrate of the SCF^{SKP2} complex. In agreement with these observations, CDK9 expression is not affected by proteasome inhibitors or by ectopic expression of SKP2, both of which dramatically affect the expression of p27. Finally, we have found no evidence that cyclin T1 levels negatively modulate CDK9 expression. It has previously been shown that endogenous CDK9 protein is stabilized by a chaperone pathway that helps form a mature, active cyclin T1/CDK9 complex (23). When CDK9 is overexpressed, a large portion of the expressed CDK9 is not stabilized and becomes rapidly degraded (10, 23; our unpublished data). Thus, when CDK9 is overexpressed, CDK9 steady-state levels do not increase more than three- to fivefold, even when the rate of synthesis of ectopically expressed CDK9 might be more than 20-fold higher than the expression level of the endogenous CDK9 (10, 23). Thus, while endogenous CDK9 is a long-lived protein, the excess monomeric CDK9 resulting from overexpression is very unstable. While this excess CDK9 might conceivably be degraded by the proteasome because it does not fold properly, we did not find evidence that the levels of SKP2 are rate limiting for stabilization of CDK9 expression. We are currently investigating the factors that limit the expression of CDK9. We suggest that if the CDK9-SCF^{SKP2} interaction has physiologic significance, SCF^{SKP2} might modulate functions unrelated to the control of CDK9 expression. In this regard, it is becoming clear that ubiquitin ligases mediate processes other than protein degradation (7). Further studies will be necessary to test this possibility.

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