

p107 inhibits G1 to S phase progression by down-regulating expression of the F-box protein Skp2

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Cell cycle progression is negatively regulated by the pocket proteins pRb, p107, and p130. However, the mechanisms responsible for this inhibition are not fully understood. Here, we show that overexpression of p107 in fibroblasts inhibits Cdk2 activation and delays S phase entry. The inhibition of Cdk2 activity is correlated with the accumulation of p27, consequent to a decreased degradation of the protein, with no change of Thr187 phosphorylation. Instead, we observed a marked decrease in the abundance of the F-box receptor Skp2 in p107-overexpressing cells. Reciprocally, Skp2 accumulates to

higher levels in *p107*^{-/-} embryonic fibroblasts. Ectopic expression of Skp2 restores p27 down-regulation and DNA synthesis to the levels observed in parental cells, whereas inactivation of *Skp2* abrogates the inhibitory effect of p107 on S phase entry. We further show that the serum-dependent increase in Skp2 half-life observed during G1 progression is impaired in cells overexpressing p107. We propose that p107, in addition to its interaction with E2F, inhibits cell proliferation through the control of Skp2 expression and the resulting stabilization of p27.

Introduction

The progression from G1 to S phase of the cell cycle is negatively regulated by a family of pocket proteins that includes the product of the retinoblastoma susceptibility gene pRb and the two closely related proteins p107 and p130. These proteins are characterized by the presence of a bipartite pocket structure (A/B domains) that is necessary for interaction with E2F transcription factors, viral oncoproteins, and other LXCXE motif-containing cellular proteins (Grana et al., 1998; Mulligan and Jacks, 1998; Classon and Dyson, 2001). Studies in cell culture systems have shown that each pRb-family member is able to cause G1 arrest when ectopically expressed (Zhu et al., 1993; Claudio et al., 1994; Weinberg, 1995). Conversely, targeted disruption of the three *Rb*-related genes in mouse embryonic fibroblasts (MEFs) leads to acceleration of G1 phase and loss of G1 arrest in response to various inhibitory signals, including low serum (Dannenberg et al., 2000; Sage et al., 2000). All

three pocket proteins are substrates of Cdks in vitro and are phosphorylated in a cell cycle-dependent manner (Grana et al., 1998; Mittnacht, 1998). Sequential phosphorylation of pRb by Cdk4/6 and Cdk2 leads to dissociation of pRb-E2F complexes, resulting in activation of E2F-dependent transcription and cell cycle progression (Weinberg, 1995; Harbour and Dean, 2000).

In mice, inactivation of the *Rb* gene results in embryonic lethality at midgestation, associated with defects in erythropoiesis and cell death in the liver and nervous system, whereas mice lacking *p107* or *p130* in the same genetic background develop normally (Mulligan and Jacks, 1998; Classon and Harlow, 2002). However, when bred on a BALB/cJ background, *p107* mutants display impaired growth and accelerated cell cycle (LeCouter et al., 1998a), whereas *p130* mutant embryos die at day 11–13 (LeCouter et al., 1998b). Double mutant mice lacking both *p107* and *p130* die soon after birth and exhibit defects in endochondral bone development (Cobrinik et al., 1996). Inactivation of *p107* or *p130* was also shown to enhance the phenotype of *Rb* mutation (Lee et al., 1996; Lipinski and Jacks, 1999). All these observations indicate that pRb, p107, and p130

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Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA.

have both overlapping and unique cellular and developmental functions.

Despite their resemblance, biochemical studies have revealed significant differences in the regulation and properties of individual pRb-family members. One clear distinction is their expression pattern during cell cycle progression (Grana et al., 1998; Nevins, 1998). Whereas the levels of pRb protein are relatively steady throughout the cell cycle and in quiescent cells, the expression of p107 and p130 vary considerably. p107 levels are low in G0 and accumulates during cell cycle reentry, whereas the levels of p130 are high in quiescent cells and drops upon growth factor stimulation. pRb-family proteins also differ in their ability to interact with the various members of the E2F family. Whereas pRb interacts with E2F1-4, p107 and p130 associate with E2F4 and E2F5 (Dyson, 1998; Trimarchi and Lees, 2002). In addition, several observations suggest that p107 and p130 are more closely related to one another than to pRb. The two proteins contain a unique motif in the spacer region that mediates binding to cyclin A- and E-Cdk2 complexes (Classon and Dyson, 2001). The biological consequence of this interaction is unclear.

Another important regulator of the G1/S transition is the Cdk inhibitor p27, which negatively regulates the activity of cyclin-Cdk2 complexes (Hengst and Reed, 1998; Sherr and Roberts, 1999). Levels of p27 are high in growth-arrested cells, and decline upon mitogenic stimulation as a result of increased proteolysis. Work by several groups have implicated the SCF^{Skp2} E3 ligase in the ubiquitin-mediated degradation of the Cdk inhibitor p27 (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). Consistent with these findings, targeted inactivation of the *Skp2* gene results in accumulation of p27 and cyclin E, and causes various cell cycle defects (Nakayama et al., 2000). More recent studies have shown that Skp2 also targets p130 for degradation (Tedesco et al., 2002; Bhattacharya et al., 2003) and participates in the regulation of Myc protein stability and activity (Kim et al., 2003; von der Lehr et al., 2003), further highlighting its central role in cell cycle control.

The generally accepted view is that pRb-family proteins negatively regulate cell proliferation by binding to E2F transcription factors, resulting in transcriptional inhibition or active repression of genes required for G1 to S phase progression. However, several studies indicate that interaction with E2F is not sufficient to explain the inhibitory action of pRb (Zhu et al., 1993; Welch and Wang, 1995) or p107/p130 (Smith and Nevins, 1995; Zhu et al., 1995; Castano et al., 1998; Gaubatz et al., 2000) on the cell cycle. Moreover, pRb-family proteins have been reported to interact with more than 100 different cellular proteins and to modulate the activity of several of these (Morris and Dyson, 2001). In the present study, we report that p107 negatively regulates expression of the F-box protein Skp2 in fibroblasts, resulting in the accumulation of p27. We provide evidence that p107 promotes the degradation of Skp2 by the proteasome. Our results identify a novel mechanism by which p107 may inhibit G1 progression through stabilization of the cell cycle inhibitor p27.

Results

Overexpression of p107 in Rat1 cells delays S phase entry

We have established a rat fibroblast cell line overexpressing p107 (Makris et al., 2002). As previously reported (Beijersbergen et al., 1995), we observed that expression of endogenous as well as ectopic p107 is cell cycle regulated (Fig. 1 A). We first examined the impact of deregulated expression of p107 on the proliferation rate of Rat1 cells. As expected, Rat1-p107HA cells proliferated much slower than parental Rat1 cells (unpublished data). To analyze the effect of p107 on the kinetics of cell cycle reentry, cells were synchronized in G0/G1 by contact inhibition and then replated at low density to allow synchronous reentry into the cell cycle. BrdU incorporation studies indicated that the percentage of cells in S phase is reduced at each time point in Rat1-p107HA cells (Fig. 1 B). After 36 h, ~90% of Rat1-p107HA cells had entered S phase, confirming that the whole population of cells is cycling. Consistent with these results, flow cytometry analysis revealed that the progression of Rat1-p107HA cells from G0 to S phase is considerably delayed as compared with Rat1 cells (Fig. 1 C). No significant number of sub-G1 cells (Fig. 1 C) or of TUNEL-positive cells (unpublished data) was observed in either contact-inhibited or exponentially proliferating Rat1-p107HA cells, indicating that p107 overexpression does not increase cell death.

Inhibition of Cdk2 activity and accumulation of p27 in Rat1 cells overexpressing p107

To investigate the molecular basis of the inhibitory action of p107 on the cell cycle, we monitored the expression and activity of key regulators of G1 phase progression. No difference in the levels of Cdk2 and Cdk4 catalytic subunits was observed between Rat1 and Rat1-p107HA cells (Fig. 2 A). The expression of cyclin D1 and cyclin A was also comparable in the two cell lines. However, the abundance of cyclin E1 was markedly increased in Rat1-p107HA cells. We also examined the expression of the Cip/Kip inhibitors p27 and p57^{Kip2}, as p21^{Cip1} is not expressed in Rat1 cells (Allan et al., 2000). Progression of Rat1 cells into the cell cycle was associated with a progressive and dramatic reduction of p27 levels in Rat1 cells. However, in Rat1-p107HA cells, the serum-mediated down-regulation of p27 was strongly inhibited (Fig. 2 A).

The activity of Cdk2 complexes is required for G0 to S phase progression in mammalian cells. We asked whether or not overexpression of p107 interferes with the enzymatic activity of Cdk2. As shown in Fig. 2 B, the enzymatic activation of Cdk2 by serum was markedly attenuated (threefold decrease at 21 h) in Rat1-p107HA cells. Because of the ability of p107 to interact with cyclin E- and A-Cdk2 complexes and to inhibit Cdk2 enzymatic activity in vitro (Castano et al., 1998), we investigated whether or not overexpression of p107 could lead to sequestration of cyclin-Cdk2 complexes. However, we observed that raising the levels of p107 does not result into increased formation of p107-cyclin-Cdk2 complexes, likely because p107 competes with the high expression levels of p27 (Fig. 2 C). To determine if the higher abundance of p27 ob-

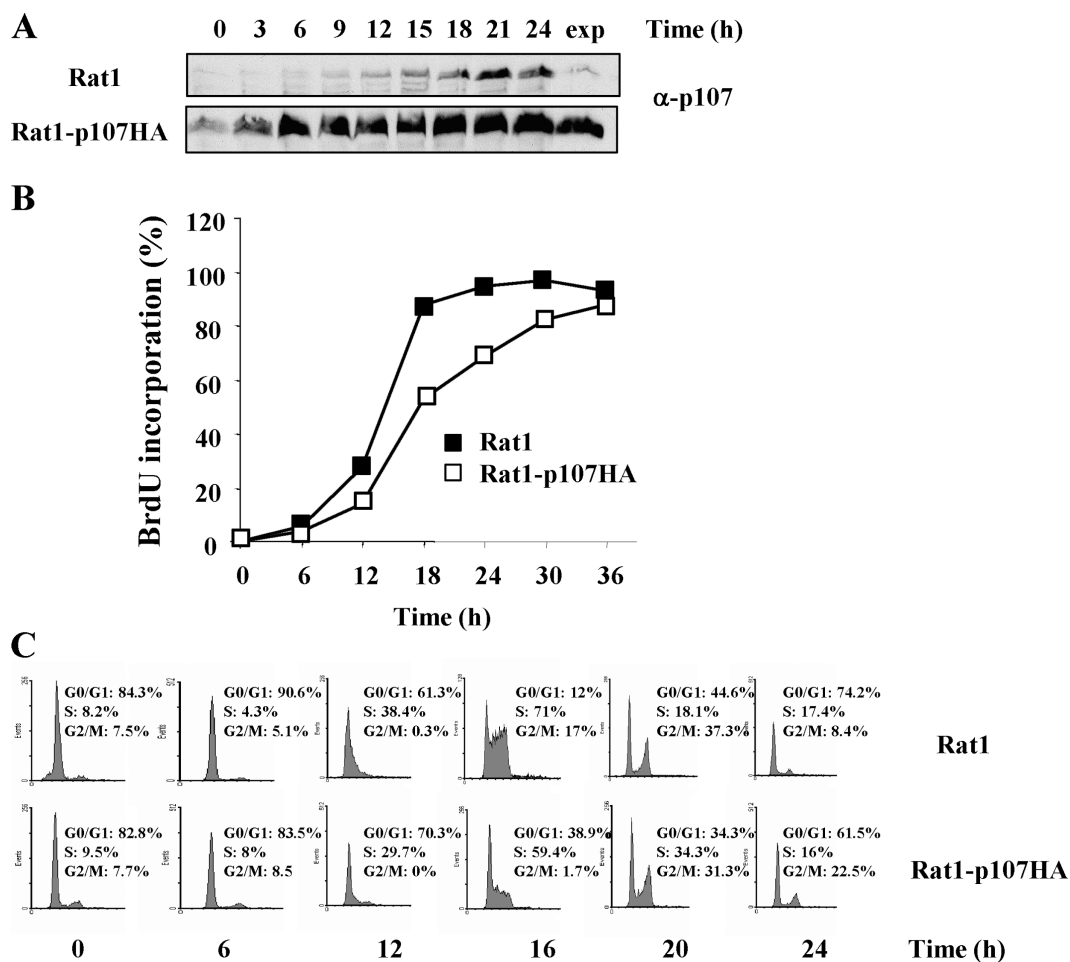


Figure 1. Overexpression of p107 delays S phase entry in Rat1 fibroblasts. Parental Rat1 and Rat1-p107HA cells were synchronized in G0/G1 by contact inhibition and replated at low confluence in serum-containing medium to allow cell cycle reentry. (A) The expression of p107 was monitored by immunoblot analysis. (B) Cells were plated on glass coverslips and the percentage of cells in S phase was analyzed by BrdU incorporation. (C) Cell cycle distribution was monitored by FACS analysis.

served in these cells was responsible for the inhibition of Cdk2 activation, we monitored the association of p27 with Cdk2 in the two cell lines. Although the amount of p27 bound to Cdk2 decreased gradually during G1 progression of Rat1 cells, the inhibitor remained tightly associated with Cdk2 in cells overexpressing p107 (Fig. 2 D). We also verified if Cdk2 and cyclin E1 were correctly assembled into complexes in p107-overexpressing Rat1 cells. Consistent with the higher expression of cyclin E1 observed in these cells, Rat1-p107HA cells were found to contain more assembled cyclin E1–Cdk2 complexes than their parental counterpart (Fig. 2 E). These results suggest that the increased abundance of p27 in Rat1-p107HA cells is responsible, at least in part, for the inhibition of Cdk2 activity.

To establish a causal relationship between p27 stabilization and the inhibitory effect of p107 on G1 to S phase progression, we used RNA interference to reduce the expression of p27 in Rat1-p107HA cells. Exponentially proliferating cells were transfected with control or p27 small interfering RNAs (siRNAs), and the percentage of S phase cells was determined. As shown in Fig. 2 F, down-regulation of p27 increased by twofold the number of Rat1-p107HA cells that progress to S phase.

p27 is stabilized in Rat1 cells overexpressing p107

One of the key mechanisms involved in the regulation of p27 abundance is its proteolysis by the ubiquitin-proteasome pathway (Pagano et al., 1995). Therefore, we evaluated the effect of p107 overexpression on the turnover of p27 by pulse-chase experiments (Fig. 3 A). The degradation rate of labeled p27 was clearly reduced in Rat1-p107HA cells (half-life 6–8 h) as compared with Rat1 cells (half-life 3–4 h) upon exposure to serum. These results were confirmed by cycloheximide-chase experiments (Fig. 3 B). Interestingly, we observed that p27 was more stable in Rat1-p107HA cells whether cycloheximide was added to quiescent cells or after 8 or 20 h of release from growth arrest.

Targeting of p27 for ubiquitination by the SCF^{Skp2} E3 ligase and subsequent degradation by the proteasome is dependent on phosphorylation of the protein at Thr187 by cyclin E–Cdk2 (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). In addition, efficient p27 ubiquitination requires formation of a trimeric complex with the cyclin and Cdk subunits (Montagnoli et al., 1999). Our results suggest (Fig. 2, D and E) that cyclin E1–Cdk2–p27 complexes are formed in Rat1-

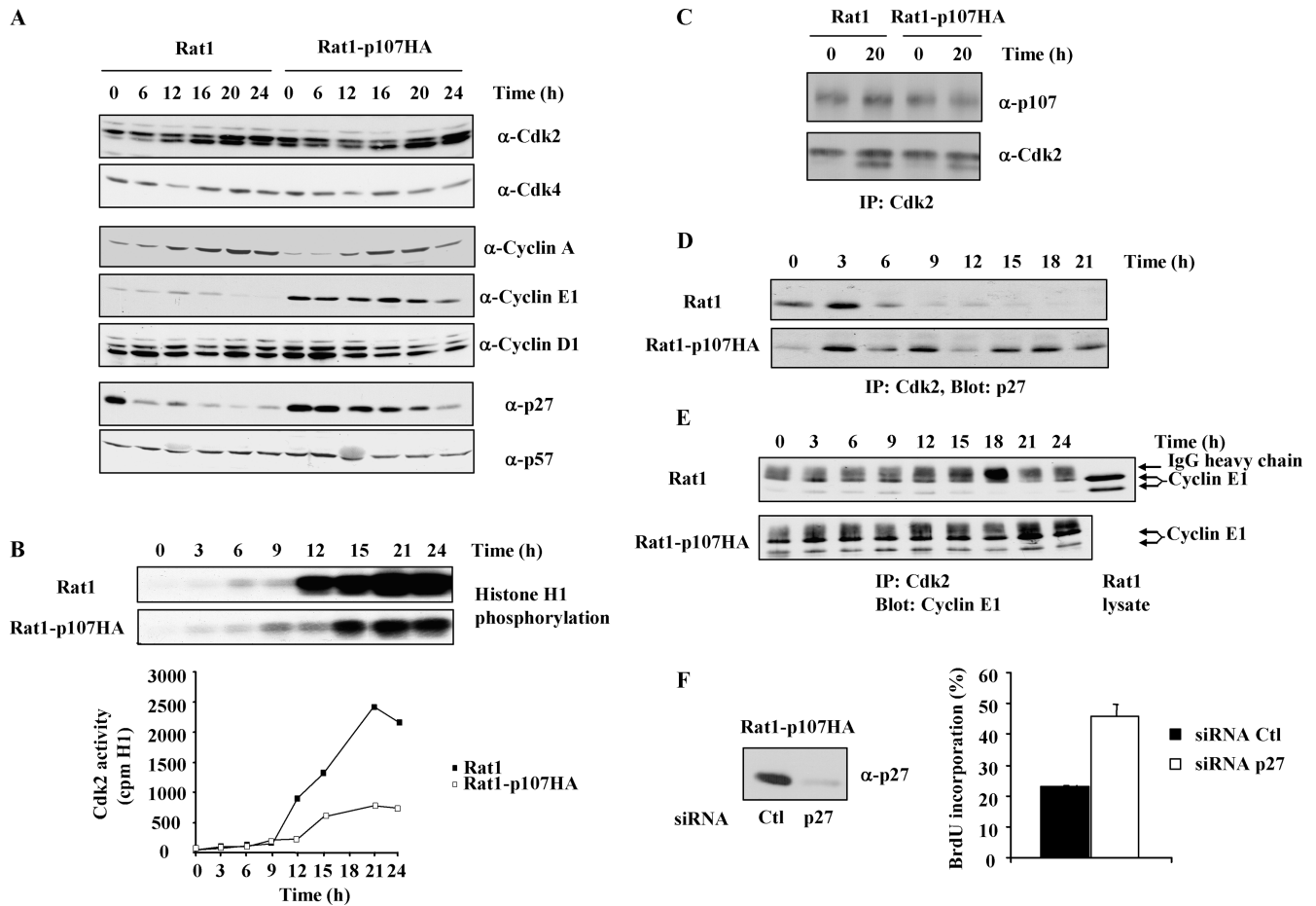


Figure 2. Overexpression of p107 prevents serum-mediated down-regulation of p27 and inhibits Cdk2 activation. Cellular extracts were prepared at the times indicated after release from contact inhibition. (A) Immunoblot analysis of cell cycle regulators. (B) The enzymatic activity of Cdk2 was assayed using histone H1 as substrate. Quantification of the data is shown in the bottom panel. (C) Binding of p107 to cyclin-Cdk2 complexes. Cdk2 immunoprecipitates were analyzed by immunoblotting with anti-p107 antibody. (D) Binding of p27 to Cdk2 complexes. Cdk2 immunoprecipitates were analyzed by immunoblotting with p27-specific antibody. (E) Complex formation between Cdk2 and cyclin E1. Cdk2 immunoprecipitates were analyzed by immunoblotting with anti-cyclin E1 antibody. (F) Rat1-p107HA cells were transfected with nonrelevant (Ctl) or p27 siRNA. After 48 h, the cells were analyzed for p27 expression (left) or for BrdU incorporation (right). The percentage of cells that incorporated BrdU over the labeling period of 12 h was evaluated by fluorescence microscopy. At least 100 cells were scored in each experiment. Results are expressed as the mean \pm SEM of three independent experiments.

p107HA cells, and thus cannot explain the impaired degradation of p27 in these cells. We next determined whether or not the decrease in Cdk2 activity observed in Rat1-p107HA cells was accompanied by a concomitant reduction in Thr187 phosphorylation of p27, which may account for the stabilization of the protein. Phosphorylation of p27 on Thr187 increases when Rat1 cells progress into the cell cycle (Fig. 3 C). We observed a higher level of Thr187 phosphorylation in Rat1-p107HA cells as compared with parental cells, which reflects the abundance of the protein. At 20–24 h of mitogenic stimulation, a time where p27 is almost undetectable in parental cells, the extent of Thr187 phosphorylation still increased without significant degradation of the inhibitor in Rat1-p107HA cells (Fig. 3 C). We concluded from these results that the stabilization of p27 in p107-overexpressing cells does not result from inhibition of Thr187 phosphorylation. Recent studies have shown that phosphorylation of p27 on Ser10 allows its translocation to the cytoplasm, resulting in stabilization of the protein (Rodier et al., 2001; Ishida et al., 2002). Therefore, we also analyzed the

state of p27 phosphorylation on Ser10 using a phospho-specific antibody. No apparent difference in the time-course or stoichiometry of Ser10 phosphorylation was observed between parental Rat1 and Rat1-p107HA cells (Fig. 3 D).

p107 negatively regulates Skp2 expression

Because phosphorylation of p27 is not affected, we next analyzed the impact of p107 on the expression of SCF^{Skp2} subunits and its cofactor Cks1. As reported previously (Lisztwan et al., 1998; Carrano and Pagano, 2001; Rodier et al., 2001), serum stimulation of G0/G1-synchronized Rat1 cells induced the accumulation of Skp2 and Cks1 proteins as cells approached S phase (Fig. 4 A). However, the abundance of Skp2 was markedly decreased in Rat1-p107HA cells, whereas the levels of Cks1 and Cul1 remained similar. To exclude the possibility that the observed changes in Skp2 expression were an indirect consequence of p107 effect on cell cycle progression, Rat1 and Rat1-p107HA cells were first sorted at the different phases of

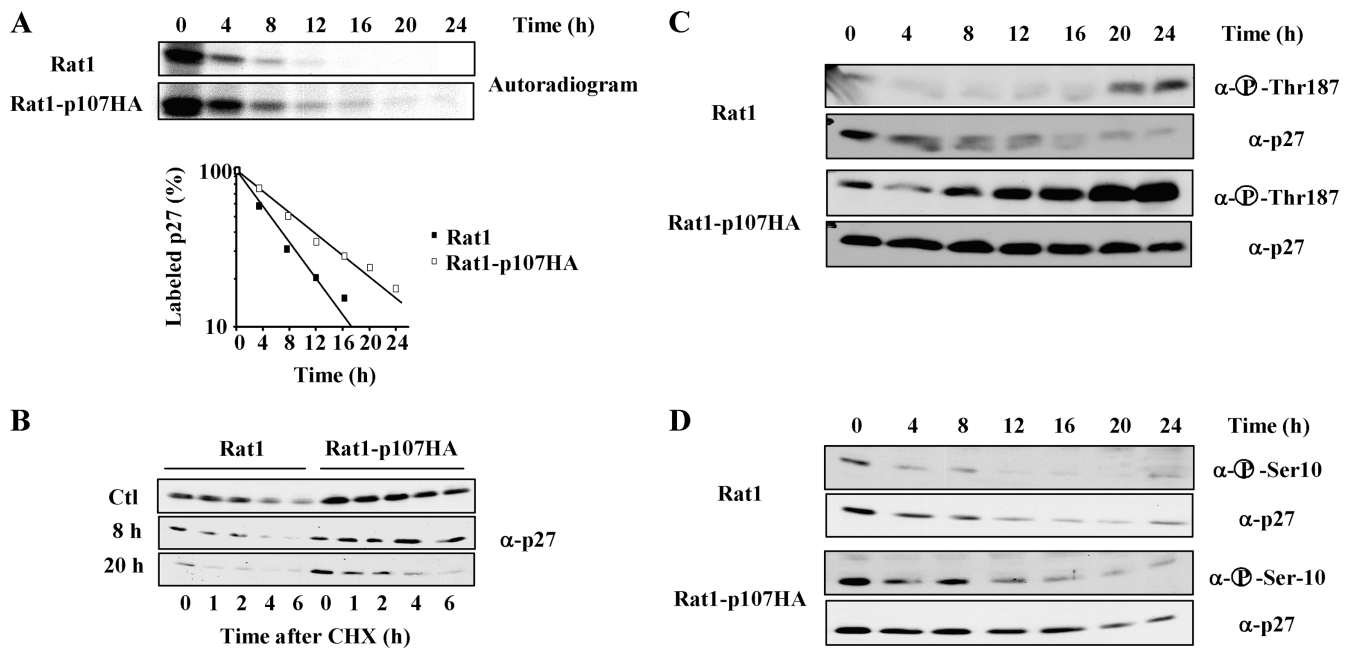


Figure 3. **Stabilization of p27 expression in Rat1 cells overexpressing p107.** (A) Quiescent cells were pulsed labeled for 2 h with [³⁵S]methionine/cysteine and chased for the indicated times in fresh medium containing 10% serum. Cell lysates were subjected to immunoprecipitation with anti-p27 antibody and the labeled p27 protein was analyzed by SDS-gel electrophoresis and fluorography. Densitometric analysis of p27 degradation rate is shown in the bottom panel. (B) Cells synchronized in G₀/G₁ (Ctl) were released from quiescence for 8 or 20 h. Then, cycloheximide (CHX) was added and the cells were harvested at the indicated intervals. The expression of p27 was analyzed by immunoblotting. (C and D) Cell extracts were analyzed by immunoblotting with antibodies specific to p27 phosphorylated on Thr187 (C) or Ser10 (D).

the cell cycle by flow cytometry before measurement of Skp2 levels. This experiment clearly showed that the expression of Skp2 is considerably decreased in all phases of the cell cycle in Rat1-p107HA cells, as compared with parental Rat1 cells (Fig. 4 B). These results suggest that Skp2 is directly regulated by p107. To determine if the effect of p107 is specific to Skp2, we monitored the expression of β TrCP, another F-box member of SCF E3 ligase complexes. The SCF β TrCP ligase is implicated in the phosphorylation-dependent ubiquitination and degradation of I κ B α in response to TNF and other signals (Rothwarf and Karin, 1999). No difference in the levels of β TrCP (Fig. 4 C) or in the turnover rate of I κ B α in response to TNF α (unpublished data) was observed between Rat1 cells and cells overexpressing p107. To confirm that the reduction in Skp2 expression is not an indirect consequence of the long-term stable overexpression of p107, we infected populations of Rat1 cells with a retrovirus encoding p107. Acute overexpression of p107 similarly resulted in the accumulation of p27 and cyclin E1 proteins, concomitant with down-regulation of Skp2 expression (Fig. 4 D).

To determine if the reduction in Skp2 abundance is causally linked to the stabilization of p27, Rat1-p107HA cells were infected with a retrovirus conferring expression of Skp2. Ectopic expression of Skp2 led to a marked decrease in cyclin E1 expression and restored the rapid down-regulation of p27 in response to serum growth factors (Fig. 4 E). We also tested if the enforced expression of Skp2 could rescue the delay in S phase entry observed in p107-overexpressing cells. Rat1-p107HA cells were transfected with either GFP or Skp2, and the reentry of cells into S phase was monitored by BrdU incor-

poration. After 20 h of serum stimulation, 50% of the cells expressing GFP had replicated their DNA (Fig. 4 F). When Skp2 was overexpressed in Rat1-p107HA cells, the percentage of BrdU-positive cells increased to 75%, a number comparable to that observed for parental Rat1 cells under these conditions (Fig. 1 B). These results suggest that the lack of Skp2 is largely responsible for the delay in S phase entry observed in cells overexpressing p107.

Skp2 is a physiological target of p107 that is important for its negative control of G₁ to S phase progression

To evaluate the physiological relevance of the aforementioned findings, we examined the regulation of Skp2 expression in *p107*^{-/-} MEFs. These cells display a twofold acceleration in doubling time (LeCouter et al., 1998a). Fig. 5 A shows that induction of Skp2 protein by serum was much greater in *p107*^{-/-} MEFs as compared with wild-type *p107*^{+/+} cells. This observation, together with the findings described in Fig. 4, strongly suggests that p107 is a negative regulator of Skp2 expression. Next, we wanted to determine if Skp2 is essential for the delay in S phase entry induced by p107. To test this idea, we monitored the incorporation of BrdU in wild-type and *Skp2*^{-/-} MEFs (Nakayama et al., 2000) transfected with a control plasmid or p107HA. In exponentially proliferating cells, the ability of p107 to decrease the number of S phase cells was completely suppressed in *Skp2*^{-/-} MEFs (Fig. 5 B). These results argue that Skp2 is a target of p107 that mediates, at least in part, its negative regulatory effect on cell cycle progression.

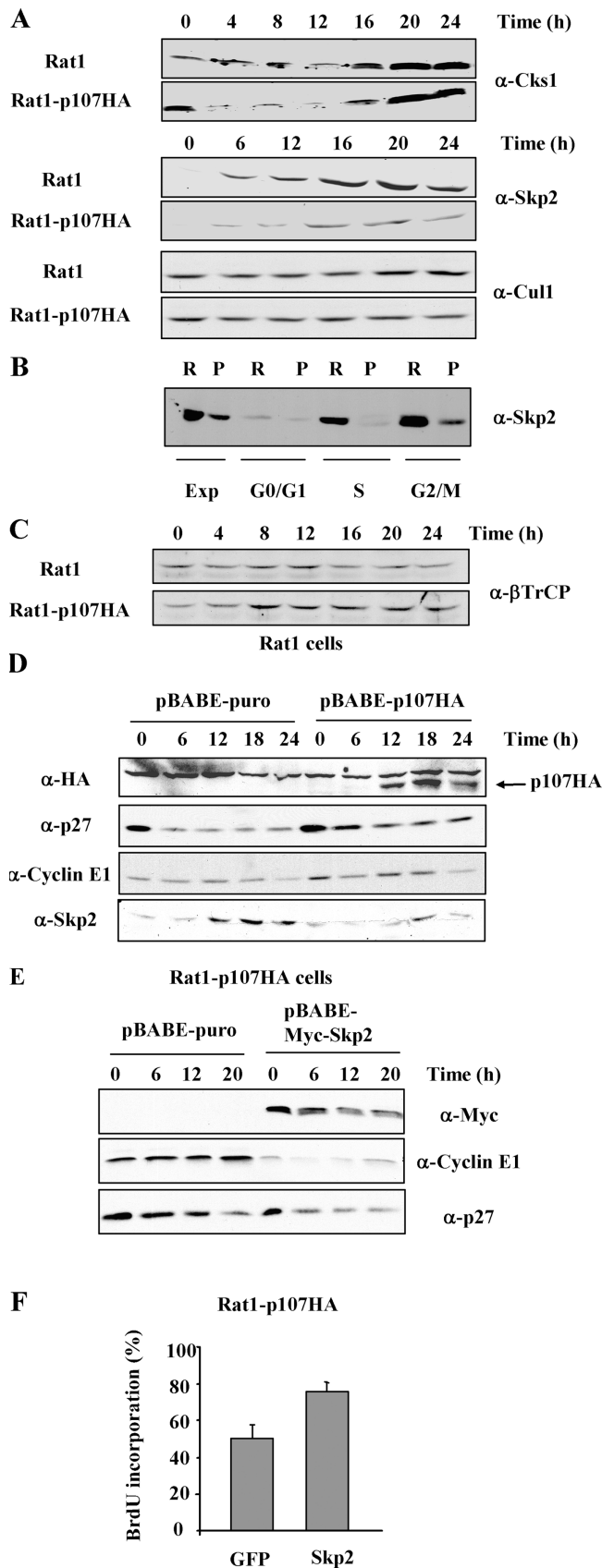


Figure 4. **p107 negatively regulates Skp2 expression.** (A) Cells were synchronized by contact inhibition and the expression of Cks1, Cul1, and Skp2 was analyzed by immunoblotting. (B) Rat1 (R) and Rat1-p107HA (P) cells were stained for DNA content with Hoescht 33342 and

p107 regulates Skp2 abundance mainly at the level of protein turnover

To investigate the mechanism by which p107 down-regulates Skp2 expression, we first measured the steady-state levels of *Skp2* mRNA. We observed that the amount of *Skp2* mRNA transiently increases when Rat1 cells progress into the cell cycle (Fig. 6 A). Quantification of the data revealed a threefold induction of *Skp2* mRNA at 12 h of mitogenic stimulation in these cells. This serum-stimulated increase in *Skp2* mRNA was significantly attenuated in cells overexpressing p107. The *Skp2* gene has been found to be up-regulated by E2F overexpression in a recent gene expression profiling study (Vernell et al., 2003). Therefore, it was of interest to test the idea that increased p107 levels may repress *Skp2* gene transcription through the formation of E2F4-DP-p107 repressor complexes. However, silencing of E2F4 by RNA interference did not restore Skp2 protein levels in Rat1-p107HA cells (Fig. 6 B), suggesting that p107 effect is independent of E2F4.

Given the relatively modest effect of p107 on *Skp2* mRNA accumulation as opposed to the drastic changes observed in Skp2 protein levels, we next analyzed the rate of Skp2 degradation at different stages of the cell cycle by cycloheximide-chase analysis. G0/G1-synchronized cells were stimulated to reenter the cell cycle with serum and cycloheximide was added at different times after stimulation. The turnover of Skp2 protein was monitored by immunoblot analysis. Serum stimulation of Rat1 cells clearly increased the stability of Skp2; the estimated half-life of the protein increased from 4.9 h in G0/G1 cells to 8.1 h in S phase-enriched cells (Fig. 6, C and D). In contrast, the effect of serum on stabilization of Skp2 was completely abolished in Rat1 cells overexpressing p107. When measured after 18 h of serum stimulation, the half-life of Skp2 was estimated at 3.8 h in Rat1-p107HA cells (Fig. 6 D). To further document the significance of p107 effect on Skp2 stability, we measured the half-life of ectopically expressed HA-tagged Skp2, which is independent of endogenous transcriptional control, in the two cell lines. Again, we observed an accelerated degradation of Skp2 in Rat1 cells overexpressing p107 (Fig. 6 E). From these results, we conclude that the decrease of Skp2 expression in p107-overexpressing cells mainly results from a lack of stabilization of the protein during G1 to S phase progression and, to a minor extent, to a decreased mRNA accumulation.

then sorted at different phases of the cell cycle. Cells extracts were analyzed by immunoblotting with anti-Skp2 antibody. (C) Cells were synchronized as in A and the expression of βTrCP was analyzed by immunoblotting. (D) Rat1 cells were infected with a retrovirus encoding p107HA. Infected cells were selected with puromycin and synchronized as in Fig. 1. Cellular extracts were analyzed by immunoblotting with anti-HA, anti-p27, anti-cyclin E1, and anti-Skp2 antibodies. (E) Rat1-p107HA cells were infected with a Skp2-encoding retrovirus and treated as in C. Cell extracts were analyzed by immunoblotting with anti-Myc, anti-cyclin E1, and anti-p27 antibodies. (F) Rat1-p107HA cells were transiently transfected with expression vectors for GFP or Skp2. The cells were serum starved for 24 h, and then stimulated for 20 h with 10% serum in the presence of BrdU. The percentage of transfected cells that incorporated BrdU was evaluated by fluorescence microscopy. Results are expressed as the mean ± SEM of three separate experiments.

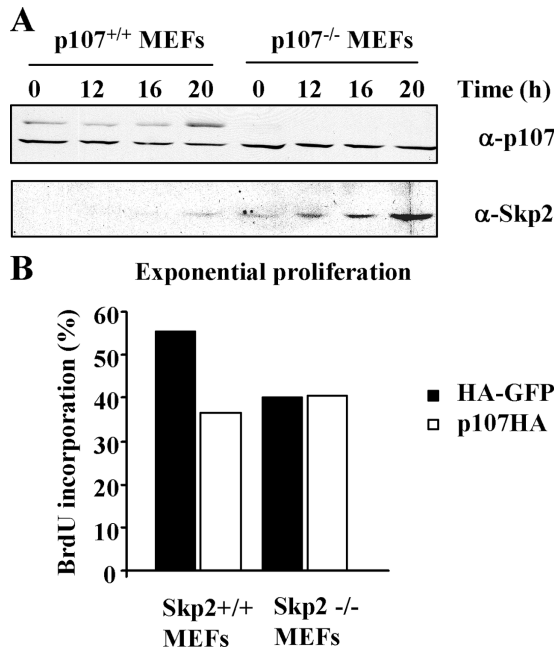


Figure 5. **Skp2 is a target of p107 action.** (A) Wild-type and *p107*^{-/-} MEFs were serum starved for 24 h and then stimulated with 10% serum for the indicated times. Cell lysates were prepared and analyzed by immunoblotting with anti-p107 and anti-Skp2 antibodies. (B) Wild-type and *Skp2*^{-/-} MEFs grown on glass coverslips were transfected with expression vectors for HA-GFP or p107HA. The cells were labeled with BrdU for 12 h and the percentage of transfected cells that incorporated BrdU was analyzed by fluorescence microscopy.

Role of Cul1 and Cdh1 in the regulation of Skp2 turnover by p107

To get some insight into the mechanism by which p107 regulates Skp2 proteolysis, we performed experiments to determine whether or not p107 expression impacts on known Skp2 degradation pathways. Early work has suggested that degradation of

Skp2 in G0/G1 cells is mediated in part by an autocatalytic mechanism involving a Cul1-based ubiquitin ligase (Wirbelauer et al., 2000). To test the role of a Cul1-based E3 ligase pathway, we used a 293 cell line conditionally expressing a Cul1 deletion mutant (Cul1-N252) that lacks the binding site for Roc1 and Cdc34 (Piva et al., 2002). Expression of this mutant is predicted to sequester Skp1-F box protein complexes and interfere with SCF-dependent degradation. Indeed, doxycycline induction of Cul1-N252 expression significantly increases the steady-state abundance of p27 in these cells (Fig. 7 A). We found that overexpression of Cul1-N252 leads to a modest accumulation of ectopically expressed HA-Skp2 (Fig. 7 A), associated with an increase in the half-life of the protein (not depicted). Enforced expression of p107 markedly down-regulated ectopic HA-Skp2, whether or not the cells were induced to express Cul1-N252 mutant (Fig. 7 A). Cycloheximide-chase experiments confirmed that the reduction in Skp2 levels was correlated with an accelerated degradation of the protein (Fig. 7 B). These results argue against the involvement of a Cul1-based ubiquitin ligase in mediating the effect of p107 on Skp2 turnover.

More recently, it has been reported that Skp2 is targeted for ubiquitination and destruction in G1 by the anaphase-promoting complex/cyclosome (APC/C) and its activator Cdh1 (Bashir et al., 2004; Wei et al., 2004). To analyze the requirement of APC/C in p107-promoted Skp2 degradation, we used RNA interference to knockdown the expression of Cdh1 in HeLa cells. Similar to the results obtained in Rat1 and 293 cells, we first documented that overexpression of p107 significantly reduces the steady-state amounts of HA-Skp2 in HeLa cells (Fig. 7 C). As expected, silencing of Cdh1 expression with siRNA resulted in the accumulation of Skp2 in exponentially proliferating cells (Fig. 7 C). However, enforced expression of p107 was still able to decrease the levels of ectopic Skp2 in Cdh1-depleted cells (Fig. 7 C), and this effect was associated with the destabilization of the protein (Fig. 7 D). Cdh1

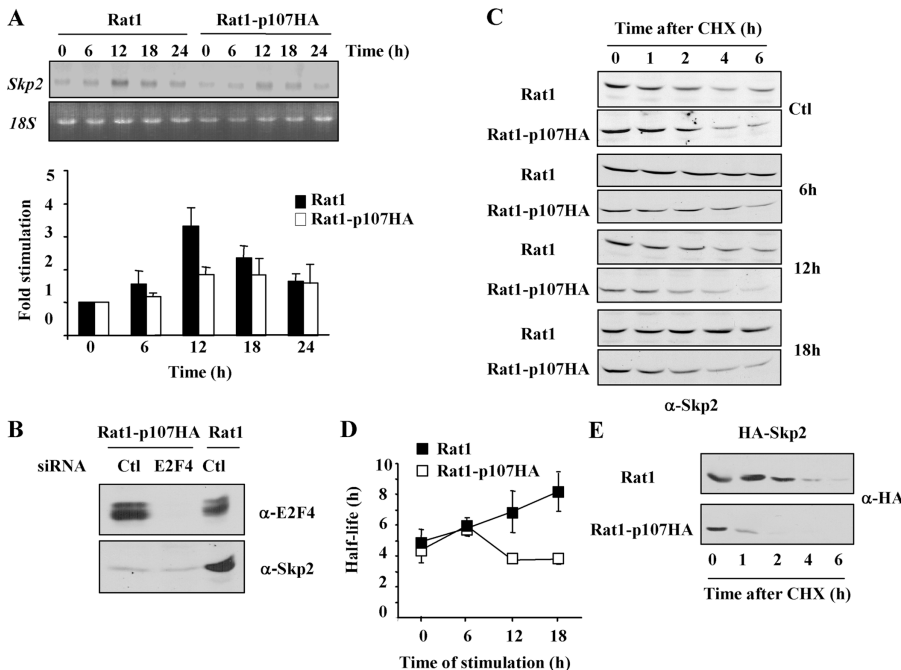
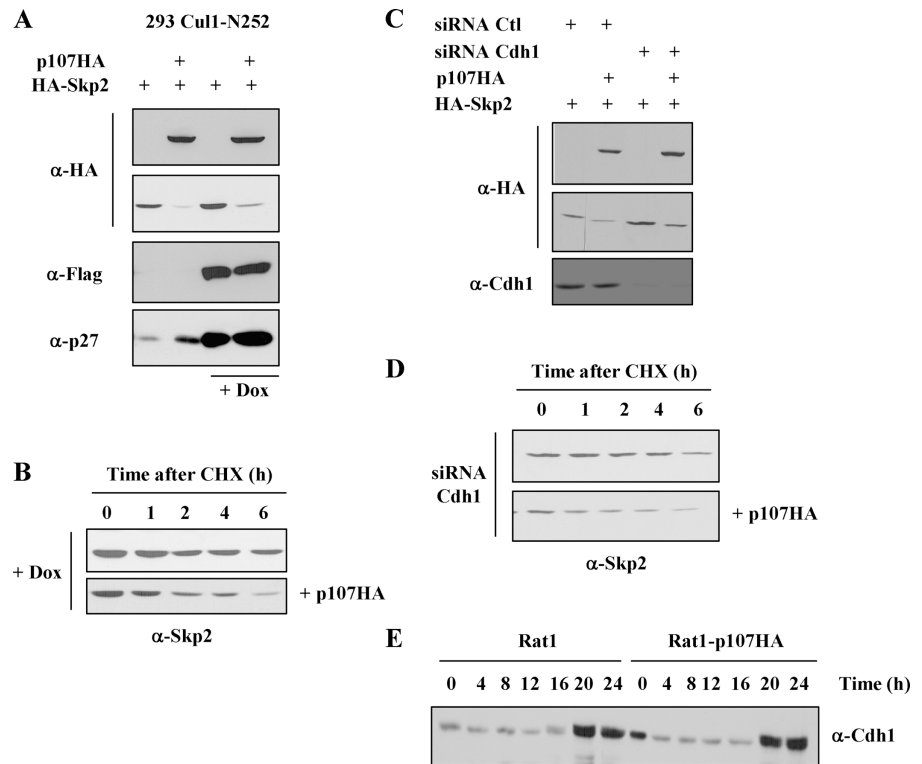


Figure 6. **Effect of p107 overexpression on the induction of Skp2 mRNA and Skp2 turnover.** (A) Northern blot analysis of *Skp2* mRNA expression. The bottom panel represents the densitometric analysis of four distinct experiments (mean \pm SEM). (B) Rat1 cell lines were transfected with nonrelevant (Ctl) or E2F4 siRNA. After 48 h, the expression of E2F4 and Skp2 was monitored by immunoblotting. (C) Cycloheximide-chase analysis of Skp2 turnover. Cycloheximide was added at different times after replating of the cells. Cell extracts were prepared at the indicated intervals and the expression of Skp2 was analyzed by immunoblotting. Different exposure times are shown to visualize the low levels of Skp2. (D) Densitometric analysis of Skp2 degradation rate at different stages of the cell cycle. Half-life values represent the mean \pm SEM of four different experiments as exemplified in C. (E) Rat1 and Rat1-p107HA cells were transfected with HA-tagged Skp2. After 48 h, cycloheximide was added and the cells were harvested at the indicated intervals. The expression of ectopic Skp2 was analyzed by immunoblotting with anti-HA antibody.

Figure 7. Role of Cul1 and Cdh1 in p107-induced Skp2 degradation. (A) 293 Cul1-N252 cells were transiently transfected with expression vectors for HA-Skp2 and p107HA. After 24 h, the expression of Flag-tagged Cul1-N252 was induced by doxycycline for 18 h. Cell extracts were analyzed by immunoblotting with anti-HA, anti-Flag, and anti-p27 antibodies. (B) Exponentially proliferating 293 Cul1-N252 cells processed as in A were treated with cycloheximide for the indicated times. The expression of ectopic Skp2 was analyzed by immunoblotting with anti-HA antibody. Different exposure times are shown to compare the half-lives of HA-Skp2. (C) HeLa cells were transfected with nonrelevant (Ctl) or Cdh1 siRNA. After 20 h, the cells were transfected with expression plasmids for HA-Skp2 and p107HA. Cell extracts were analyzed by immunoblotting with anti-HA and anti-Cdh1 antibodies. (D) HeLa cells processed as in C were treated with cycloheximide for the indicated times and the expression of HA-Skp2 was monitored by immunoblotting. Different exposure times are shown. (E) Cells were synchronized by contact inhibition and the expression of Cdh1 was analyzed by immunoblotting.



was shown to be phosphorylated in S, G2, and early M phase by cyclin A-Cdk2, and this phosphorylation is thought to contribute to APC/C^{Cdh1} inactivation by dissociating Cdh1 from the APC/C complex (Peters, 2002). We monitored the phosphorylation status of Cdh1 in Rat1 and Rat1-p107HA cells to determine if p107 overexpression could prevent the inactivation of Cdh1. As shown in Fig. 7 E, although the phosphorylation of Cdh1 was slightly delayed in Rat1-p107HA cells, the extent of phosphorylation was comparable in both cell lines at 20 h, as revealed by the presence of a slower-migrating form (Kramer et al., 2000). Comparable results were obtained by immunoprecipitating Cdh1 from ³²P-labeled cells (unpublished data). These results suggest that p107 regulates proteolysis of Skp2 in part by a mechanism independent of Cdh1 activity.

Skp2 does not target p107 for degradation

Skp2 mediates the ubiquitin-dependent degradation of various cell cycle regulatory proteins, including the pRb-family member p130 (Tedesco et al., 2002). Interestingly, we found that p107 and Skp2 physically associate upon coexpression in NIH 3T3 cells (unpublished data). To determine if p107 is a target of the SCF^{Skp2} complex, we monitored the expression of p107 in wild-type and Skp2^{-/-} cells during cell cycle progression. No difference in p107 protein levels was observed between the two cell lines (Fig. 8).

Discussion

In this work, we show that overexpression of the pRb-family protein p107 inhibits mitogenic stimulation of Cdk2 and delays S phase entry in fibroblasts. The inhibition of Cdk2 activity does

not result from a defect in the synthesis or assembly of cyclin E and Cdk2 into complexes, or to the sequestration of cyclin E- and A-Cdk2 complexes by p107 (Fig. 2). Indeed, no increase in the binding of p107 to Cdk2 could be observed in p107-overexpressing cells. Instead, it correlates with the accumulation of p27 and its tight binding to cyclin E-Cdk2 complexes. Importantly, we provide compelling evidence that the stabilization of p27 is due to the ability of p107 to directly down-regulate expression of the F-box protein Skp2. This mechanism is supported by several observations. First, no significant change in the stoichiometry of p27 phosphorylation on Thr187 was observed in p107-overexpressing cells, despite the marked inhibition of Cdk2 activity, suggesting that the accumulation of p27 is the cause rather than the consequence of Cdk2 inactivation. Loss of cell adhesion similarly results in the accumulation of phosphorylated p27, consequent to the absence of Skp2 expression (Carrano and Pagano, 2001). Second, enforced expression of Skp2 in p107-overexpressing cells restores normal p27 degradation and S phase entry. Third, embryonic fibroblasts isolated from p107^{-/-} mice display a marked accumulation of Skp2. Fourth, targeted disruption of Skp2 results in a similar accumulation of cyclin E and p27 as that observed in Rat1-p107HA cells (Nakayama et al., 2000). In agreement with the latter work, no change in the abundance of E2F1 was observed in cells overexpressing p107 (unpublished data). Finally, Rat1-p107HA cells exhibit a flat morphology and enlarged size as compared with parental Rat1 cells (unpublished data). A similar morphological change was described in Rat1 cells infected with p27-encoding retrovirus (Vlach et al., 1996) and, most interestingly, in Skp2-deficient fibroblasts (Nakayama et al., 2000). Thus, we conclude that p107 is a negative regulator of Skp2 expression.

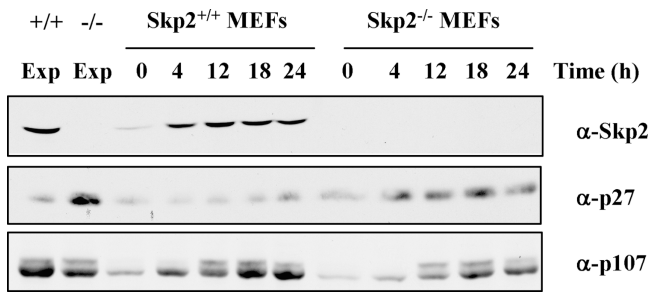


Figure 8. **Inactivation of *Skp2* does not affect p107 stability.** Wild-type and *Skp2*^{-/-} MEFs were serum starved for 24 h and stimulated with 10% serum for the indicated times. Cell lysates were prepared and analyzed by immunoblotting with antibodies to Skp2, p27, and p107.

The precise mechanisms underlying the regulation of Skp2 expression during the cell cycle are not fully understood. The levels of Skp2 protein are undetectable or low in G0/early G1, increase gradually during progression to S and G2 phases, and then decline abruptly in late M phase (Lisztwan et al., 1998; Wirbelauer et al., 2000; Carrano and Pagano, 2001; Rodier et al., 2001; Bashir et al., 2004; Wei et al., 2004). The induction of Skp2 is associated with the cell cycle-dependent accumulation of *Skp2* mRNA (Zhang et al., 1995; Carrano and Pagano, 2001; Imaki et al., 2003) and the stabilization of the protein (Wirbelauer et al., 2000; Bashir et al., 2004; Wei et al., 2004). It has been suggested that the transcriptional induction of *Skp2* mRNA results from the cell cycle-dependent binding of GA-binding protein to the *Skp2* promoter (Imaki et al., 2003). Cell adhesion to the extracellular matrix is also required for the accumulation of *Skp2* mRNA (Carrano and Pagano, 2001). Two recent studies have provided an important insight into the cell cycle dependence of Skp2 degradation, by showing that Skp2 is targeted for ubiquitination and destruction by the APC/C^{Cdh1} ubiquitin ligase (Bashir et al., 2004; Wei et al., 2004). Earlier work also suggested that the rapid turnover of Skp2 in G0/G1 cells involves a Skp2-bound Cul1-based core ubiquitin ligase (Wirbelauer et al., 2000). Moreover, Skp2 was found to self-ubiquitinate in the presence of SCF components in *in vitro* ubiquitination studies (Wirbelauer et al., 2000; Wang et al., 2004). Thus, expression of Skp2 appears to be controlled by multiple transcriptional and posttranscriptional mechanisms.

In this work, we found that overexpression of p107 partially suppresses the transient increase in *Skp2* mRNA detected as cells progress into G1 phase. p107 is known to bind specifically to E2F4, which is primarily involved in the repression of E2F-responsive genes (Dyson, 1998; Trimarchi and Lees, 2002). Of note, the *Skp2* gene was recently shown to be up-regulated upon E2F induction in U-2 OS cells (Vernell et al., 2003), although other DNA microarray experiments failed to identify *Skp2* as an E2F target. Here, we showed that complete silencing of E2F4 has no impact on Skp2 levels in p107-overexpressing Rat1 cells, thereby suggesting that *Skp2* is not a bona fide E2F4 target gene. Consistent with these findings, microarray analysis of anti-E2F4 chromatin immunoprecipitates failed to identify the Skp2 promoter among the target genes (Ren et al., 2002; Weinmann et al., 2002).

Our results suggest that repression of *Skp2* transcription is unlikely to be the principal mechanism by which p107 down-regulates Skp2 expression. We found that increasing the levels of p107 completely inhibits the stabilization of Skp2 protein that occurs during G1 to S phase progression. This depends on a direct effect of p107 on Skp2 turnover as the ectopically expressed Skp2 was also found to be less stable in p107-overexpressing cells. We also observed that the L19 mutant of p107 (Zhu et al., 1995), which is defective in E2F binding, effectively promotes the degradation of Skp2, demonstrating the lack of involvement of E2F transcriptional activity in this process (unpublished data). Various mechanisms can be envisaged to explain the effect of p107 on Skp2 degradation. We have shown that Cul1 and Cdh1 levels are unchanged in p107-overexpressing cells. We also provide evidence that inactivation of Cul1-based ubiquitin ligases by overexpression of Cul1-N252 mutant or inactivation of APC/C^{Cdh1} by silencing of Cdh1 expression is not sufficient to render Skp2 immune to p107 overexpression, suggesting that p107 may regulate Skp2 proteolysis, at least in part, by a novel pathway. In further support of this idea, we observed that the half-life of Skp2 is much shorter in p107-overexpressing cells when measured at 18 h after serum stimulation, a time where a large proportion of cells are in S and G2/M phases and Cdh1 is inactive (Fig. 6 C, bottom panel). The steady-state levels of Skp2 were also found to be significantly lower in S and G2/M phase extracts of Rat1-p107HA cells, as compared with Rat1 cells (Fig. 4 B). Intriguingly, we found that p107 can be coprecipitated with Skp2 *in vivo* upon coexpression of the two proteins (unpublished data), but the physiological significance of this observation remains to be established. Our results do not exclude the possibility that p107 contributes in some way to the regulation of Skp2 degradation by APC/C^{Cdh1}. Indeed, we observed that the impact of p107 overexpression on Skp2 turnover was somewhat less pronounced in Cdh1-silenced cells. Additional studies are clearly warranted to clarify these issues.

The expression of Skp2 is elevated in many transformed cells (Zhang et al., 1995) as well as in various human cancers, including lymphomas (Latres et al., 2001), oral epithelial carcinomas (Gstaiger et al., 2001), and breast cancers (Signoretti et al., 2002). The overexpression of Skp2 correlates with increasing grade of the tumor. Importantly, Skp2 can cooperate with activated Ras to transform primary cells *in vitro* and *in vivo*, demonstrating its oncogenic potential (Gstaiger et al., 2001; Latres et al., 2001). These observations underscore the importance of elucidating the mechanisms that control Skp2 abundance in normal and cancer cells. Our work identifies p107 as a novel regulator of Skp2 expression. p107 levels normally increase in late G1 to reach a maximum in S and G2/M phases of the cell cycle. Interestingly, we found that inactivation of p107 in fibroblasts leads to the inappropriate expression of Skp2 during G1 to S phase progression. From these observations, it is tempting to speculate that p107 may act as a modulator that limits Skp2 accumulation in G1 to prevent premature S phase entry. p107 may also act later to control the correct timing of S phase and mitosis (Nakayama et al., 2004).

Materials and methods

Cell culture, cell synchronization, and infections

The Rat1-p107HA cell line has been described previously (Makris et al., 2002). Rat1 cells were arrested by contact inhibition for 3 d and replated at low confluence to allow cell cycle reentry. MEFs from *p107^{-/-}* mice (LeCouter et al., 1998a; provided by M. Rudnicki, Ottawa Health Research Institute, Ottawa, Canada) were grown in DME supplemented with 10% FBS and were synchronized in G₀/G₁ by serum starvation. These cells were used at passage 5. *Skp2^{-/-}* MEFs were cultured as described previously (Nakayama et al., 2000) and used at passage 2–4. Rat1-p107HA cells and *Skp2^{-/-}* MEFs were transiently transfected using FuGENE 6 reagent (Roche Diagnostics). 293T cells conditionally expressing the Cul1-N252 mutant were provided by M. Pagano (New York University School of Medicine, New York, NY) and used as described previously (Piva et al., 2002). For infection of Rat1 populations, 293 cells were cotransfected with pBabe-puro vectors and pCLAmpho (Imgenex; 2:1 ratio) by the calcium phosphate precipitation method. After 24 h, the medium was changed and the cells were cultured for an additional 24 h. Then, the viral supernatant was harvested and added to Rat1 cultures in the presence of 10 µg/ml polybrene. After 24 h, the medium was changed and the cells were cultured for an additional 24 h in fresh medium. Populations of infected cells were selected with 4 µg/ml puromycin and analyzed as indicated.

Antibodies and DNA constructs

The p27 phospho-Ser10 specific antibody was generated in collaboration with Cell Signaling Technology. Polyclonal antibodies to p107 (C18), Cks1 (FL79), p57^{Kip2} (H91), βTrCP (H300), Myc (A14), and HA (Y11) were obtained from Santa Cruz Biotechnology, Inc. mAbs to cyclin A (E-23), Cdk4 (DCS-35), E2F4 (Ab-4) and Cdh1 (DH-01), and polyclonal anti-cyclin D1 (Ab-4) were obtained from Neomarkers. The source of other antibodies has been described previously (Servant et al., 2000; Rodier et al., 2001).

The human HA-tagged p107 cDNA (Zhu et al., 1995; provided by L. Zhu, Albert Einstein College of Medicine, Bronx, NY) was ligated into the BamHI and XhoI sites of pBabe-puro. The human Skp2 cDNA (provided by H. Zhang, Yale University School of Medicine, New Haven, CT) was ligated into the EcoRI site of pcDNA3-Myc₆, pBabe-puro-Myc₆, and pcDNA3-HA expression vectors (Coulombe et al., 2004). pcDNA3-Myc₆-GFP was constructed by subcloning the PCR-amplified GFP sequence into the EcoRI-XbaI sites of pcDNA3-Myc₆. pcDNA3-HA-GFP has been described previously (Coulombe et al., 2003).

Immunoblot analysis and Cdk2 kinase assays

Cell lysis, immunoprecipitation, and immunoblot analysis were performed as described previously (Servant et al., 2000). For coimmunoprecipitation studies, cell lysates (500 µg of protein) were incubated for 4 h at 4°C with anti-Cdk2 or anti-HA antibodies cross-linked to protein A-Sepharose beads, and the precipitated proteins were analyzed by immunoblotting with p27, p107, cyclin E1, or Myc specific antibodies. The phosphotransferase activity of Cdk2 was measured by immune complex kinase assay using histone H1 as substrate (Servant et al., 2000).

Pulse-chase experiments

Pulse-chase analysis of p27 turnover was performed as described previously (Rodier et al., 2001). For cycloheximide chase, cells at different stages of the cell cycle were incubated with 100 µg/ml of cycloheximide for the indicated times. Cell lysates were prepared and the expression of p27 or Skp2 was analyzed by immunoblotting.

Cell cycle analysis and cell sorting

For BrdU incorporation studies, cells plated on glass coverslips were serum starved and then stimulated with 10% serum for the times indicated in the presence of 10 µM BrdU. The cells were fixed as described previously (Rodier et al., 2001). DNA was denatured by treatment with 2 N HCl for 10 min and staining was performed by incubating cells for 1 h at 37°C with anti-BrdU antibody, followed by incubation with FITC-conjugated anti-mouse IgG as secondary reagent. DAPI staining was performed to visualize the nuclei. Cell samples were analyzed by fluorescence microscopy (model DM RB; Leica), and the percentage of cells showing nuclear labeling for BrdU was calculated. For transfection studies, the transfected cells were first detected by immunostaining with anti-Myc or anti-HA as primary antibody and FITC-conjugated anti-rabbit IgG as secondary reagent. The DNA was then denatured and BrdU was stained as described, except that the secondary reagent was a Rhodamine-conjugated anti-mouse IgG. A minimum of 100 cells was scored for each coverslip. The re-

sults are expressed as the percentage of transfected cells showing nuclear BrdU labeling.

For cell cycle analysis, exponentially proliferating or G₀-synchronized cells allowed to reenter the cell cycle were scraped in 1 mM PBS/EDTA, fixed in cold 70% ethanol, and kept at -20°C until flow cytometry analysis. The cells were washed with PBS and incubated on ice for 30 min in PI buffer (0.1% sodium citrate, 50 µg/ml propidium iodide, and 0.2 mg/ml RNase) in the dark. Fluorescence was recorded on an flow cytometer (model epics XL; Beckman Coulter) and the cell cycle profiles were determined using the Multicycle AV software. For cell sorting experiments, the cells were stained with 7.5 µg/ml of Hoescht 33342 for 30 min at 37°C, and then sorted in the G₀/G₁, S, and G₂/M phases of the cell cycle using the MoFlo high speed cell sorter (DakoCytometry). Once sorted, the cells were lysed and analyzed as described previously (Servant et al., 2000).

RNA interference

siRNAs were purchased from Dharmacon and transfected into Rat1 or HeLa cells using Oligofectamine reagent (Invitrogen). The siRNA target sequences were 5'-CUGCCGAGAUUGGAAGAAdTdT-3' (p27), 5'-CGAGAGUGAAGGUGUCUGdTdT-3' (E2F4), and 5'-UGAGAAGU-CUCCCAGUCAGdTdT-3' (Cdh1).

Northern blot analysis

RNA isolation and Northern blot analysis was performed as described previously (Servant et al., 2000). The probe used was a 1-kb EcoRI fragment of the rat Skp2 cDNA (provided by G. Baffet, Hôpital Pontchaillou, Rennes, France).

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