Down-regulation of p27\textsuperscript{Kip1} Promotes Cell Proliferation of Rat Neonatal Cardiomyocytes Induced by Nuclear Expression of Cyclin D1 and CDK4

EVIDENCE FOR IMPAIRED Skp2-DEPENDENT DEGRADATION OF p27 IN TERMINAL DIFFERENTIATION*

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Mammalian cardiomyocytes lose their capacity to proliferate during terminal differentiation. We have previously reported that the expression of nuclear localization signal-tagged cyclin D1 (D1NLS) and its partner cyclin-dependent kinase 4 (CDK4) induces proliferation of rat neonatal cardiomyocytes. Here we show that the D1NLS/CDK4 cells, after their entry into the cell cycle, accumulated cyclin-dependent kinase inhibitor p27 in the nuclei and decreased the cyclin-dependent kinase 2 (CDK2) activity, leading to early cell cycle arrest. Biochemical analysis demonstrated that Skp2-dependent p27 ubiquitylation was remarkably suppressed in cardiomyocytes, whereas Skp2, a component of Skp1-Cullin-F-box protein ubiquitin ligase, was more actively ubiquitylated compared with proliferating rat fibroblasts. Specific degradation of p27 by co-expressing Skp2 or p27 small interfering RNA caused an increase of CDK2 activity and overrode the limited cell cycle. These data altogether indicate that the impaired Skp2-dependent p27 degradation is causally related to the loss of proliferation in cardiomyocytes. This provides a novel insight in understanding the molecular mechanism by which mammalian cardiomyocytes cease to proliferate during terminal differentiation.

Terminal differentiation of highly specialized cells such as neural cells, cardiomyocytes, and pancreatic \(\beta\) cells is an important biological process that ensures their proper mass and function in higher organism. This process is accompanied with cell cycle arrest that is a hallmark of terminally differentiated cells. However, it is poorly understood how these cells cease to proliferate or more specifically whether their cell cycle can be reactivated. Mammalian cardiomyocytes irreversibly withdraw from the cell cycle soon after birth and lose the cell proliferative activity (1–4), although it has been shown that the adult heart contains a small population of cardiac progenitors that retain the proliferation capacity (5, 6). It is recently demonstrated that cardiac progenitor cells from embryonic or bone marrow stem cells are differentiated into functional cardiomyocytes when grafted in the damaged cardiac tissue (5). In addition to these, several approaches have been made to reactivate the cell cycle of terminally differentiated cardiomyocytes. For instance, adenoviral delivery of E2F allowed these cells to enter the cell cycle. However, this could not support cell proliferation but caused apoptotic cell death (7–9). This failure of cell proliferation may be largely because of our limited knowledge of the molecular events of cell cycle arrest underlying terminal differentiation of cardiomyocytes.

We have recently reported that the nuclear expression of cyclin D1 (D1NLS)/cyclin-dependent kinase (CDK) 4 promoted the proliferation of rat neonatal cardiomyocytes in culture as well as adult rat heart in situ (10). Furthermore, there observed no induction of apoptotic cell death. This argues that the nuclear import of cyclin D1 is impaired in adult cardiac cells and these cells could be allowed to enter the cell cycle once this step is bypassed. This may illustrate one of the molecular features of adult cardiomyocytes that cease to grow after terminal differentiation. However, our knowledge is still limited regarding the molecular mechanism by which the proliferation of adult cardiomyocytes is suppressed or how it is reactivated. It is also unknown whether there is a cell cycle barrier other than the nuclear import of cyclin D1.

Progression of the mammalian cell cycle is regulated with a combination of positive and negative regulators (11). It is activated by a family of cyclins and CDKs. During the G1 phase, cyclin D1 and other D-type cyclins accumulate in nuclei and assemble with their catalytic partners, CDK4 and CDK6. For example, the cyclin D1-CDK4 complex phosphorylates and in-

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The abbreviations used are: D1NLS, nuclear localization signal-tagged cyclin D1; CDK, cyclin-dependent kinase; SCF, Skp1/Cullin/F-box; siRNA, small interfering RNA; APC/Cdh1, anaphase-promoting complex/cyclosome and its activator Cdh1.
activates the retinoblastoma protein (Rb) and sequesters CDK inhibitors, thereby promoting cell cycle progression through the G1 to S phase. On the other hand, the CDK inhibitors, such as CIP/KIP family proteins, negatively regulate progression of the cell cycle by inhibiting the activity of cyclin-CDK complexes. The CIP/KIP family proteins, including p21 and p27, suppress the activities of cyclin A/CDK2 and cyclin E/CDK2, respectively, and mediate the exit from the cell cycle. The level of p27 increases in quiescent non-proliferating cells and decreases on entry into the cell cycle and is controlled by the rate of degradation in both cytoplasm and nuclei. The degradation of p27 in cytoplasm is associated with the transi-
tion of cells from the G0 to G1 phase, whereas nuclear degra-
dation occurs during S and G2 phases. In the latter, p27 is phosphorylated at threonine 187 and recruited to the F-box protein Skp2 of the SCFSkp2 complex. This is followed by mul-
tiple ubiquitylation and degradation through the 26 S protea-
some. Among this cascade reaction, the recognition of p27 by Skp2 is specific and plays a central role in p27 degradation. Therefore, p27 degradation is essential for cells to undergo cell cycle progression (12–19). In the developing heart, p27 is de-
creased on entry into the cell cycle and is controlled by

The Role of p27 in Proliferating D1NLS/CDK4 Cardiomyocytes

FIG. 1. CDK2 activity was suppressed and p27 accumulated in cardiomyocytes after cell cycle entry by D1NLS/CDK4. Rat neonatal cardiomyocytes were infected with a combination of AdD1NLS and AdCDK4 as described under “Experimental Procedures.” A, at each time indicated, cell extracts were immu-

Experimental Procedures

Reagents—Antibodies used in this study were as follow: mouse mono-
clonal anti-cyclin D1 (Ab-3, Oncogene Science), anti-sarcomeric actin (M0874, DAKO), anti-proliferating cell nuclear antigen (sc-056), and rabbit polyclonal anti-p21 (sc-6246), anti-p27 (sc-528), anti-Skp2 p45 (sc-
7164), anti-CDK4 (sc-260), and anti-CDK2 (sc-163) from Santa Cruz Bio-
technology, Santa Cruz, CA. Other biochemicals used were reagent grade.

Cell Culture—Rat neonatal cardiomyocytes were isolated and cul-
tured as previously described (10, 22). Heart ventricles were isolated from 3-day-old postnatal Sprague-Dawley rats, trisected, and then dis-
gested 4 times with 1 mg/ml collagenase type II (Worthington) in Ads buffer (20 mM Hepes-KOH, pH 7.35, 116 mM NaCl, 5.4 mM KCl, 1 mM
NaH2PO4, 0.8 mM MgSO4, and 5 mM glucose) at 37 °C for 20 min. The dispersed cells were washed once by Ads buffer and then purified by cen-
trifugation through a discontinuous Percoll gradient of 1.050, 1.060, and 1.082 g/ml, respectively. The cardiac cells at the interface between
1.060 and 1.082 g/ml were collected and plated on a 60-mm dish (2–3
105 cells) or 25-mm collagen-coated coverslips (2–3 x 104 cells) in
minimum essential medium supplemented with 5% calf serum, 100
units/ml penicillin, and 100 μg/ml streptomycin. Cardiomyocytes were
cultured at 37 °C for 24 h in humidified air with 5% carbon dioxide, after

Adenoviruses and Their Gene Transfer—Adenoviruses for expressing
nuclear localization signal-tagged cyclin D1 (Ad-D1NLS), CDK4 (Ad-
CDK4), and Skp2 (Ad-Skp2) were as described (10, 23). Viruses were
propagated in 293 cells, and the virus stocks were prepared as described
(10, 22). Virus titer was determined by an indirect immunofluorescent

In the present study, we examined the cell cycle progression of
rat neonatal cardiomyocytes that were forced to enter the cell cycle by nuclear expression of D1NLS and its partner CDK4 (10). It was shown that D1NLS/CDK4 cardiac cells ceased to proliferate after only one or two cell cycles and accumulated the CDK inhibitor p27 in the nuclei. The in vitro p27 ubiquitylation was remarkably suppressed in cardiomyocytes, whereas that of

Skp2 was up-regulated. We further showed that knockdown of p27 or overexpression of Skp2 specifically degraded p27 and accelerated proliferation of D1NLS/CDK4 cardiomyocytes.
assay using anti-72K antibody. Cardiomyocytes were infected at the indicated multiplicity of infection in serum-free minimal essential medium for 60 min with brief agitation every 15 min, and then the medium was replaced with culture medium.

Construction of Adenovirus Vector Encoding p27 SiRNA—Oligonucleotides for expressing stem-loop RNAs that target three different regions of rat p27 were ligated with human U6 promoter and the resulting transcription unit of the U6 promoter was subcloned into the SwaI site of the E1-deleted region of cassette cosmid vector pAxcw (Adenovirus Expression Vector Kit, TaKaRa BIO). Adenoviral vector constructs were then prepared as in the protocol from Takaka. Oligonucleotide sequences used were: 5'-CACCCGTAGGAGCTTCTGTAACGCTGTTGCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAAAAATGGAGAATCTTCTGTTGAAGAAAGAATCTTCTGCCTTTTTT-3' and 5'-GCATAA
fractionated on a formaldehyde/agarose gel, transferred to a Hybond-N membrane, and hybridized to random-primed cDNA probes for the mouse p27 and skp2 genes as described (22). The membrane was exposed with a BAS 2500 Bioimage analyzer (Fuji Film Co., Tokyo, Japan). DNA fragments (100 ng) for these cDNAs were radiolabeled with 50 Ci of [32P]dCTP (600 Ci/mmol, Amersham Bio- sciences) using a random primer labeling kit from Takara and used as probe for RNA preparation and Northern blot analysis.

In Vitro Ubiquitylation Assay—Cells were treated in lysis buffer (20 mM Hepes-KOH, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5% Nonident P-40, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotonin, and 25% glycerol) on ice for 30 min, and cell debris was removed by centrifugation at 15,000 rpm for 20 min. Cell extract (50 μg of protein) was incubated with substrates in 15 μl of buffer of 40 mM Hepes-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM dithiothreitol, 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, and 1.5 mM ATP containing 50 ng of Uba1, 100 ng of UbE1A, 3 μg of glutathione S-transferase-ubiquitin. After incubating at 26 °C for 30 min, reaction products were separated by SDS-PAGE and analyzed by Western blot with the indicated antibody. As substrates, FLAG-p27 or -Skp2 were prepared using 1 μg of pcDNA3Flag-p27 or pcDNA3Flag-skp2 (23) and 25 μl of rabbit reticulocyte lysate (TNT kit from Promega) according to its protocol. The in vitro p27 ubiquitylation activity was also assayed after immunoprecipitation of cell extracts with 0.4 μg of anti-Skp2 antibody.

Immunocytological Study and Cell Cycle Analysis—To examine the expression of p27 in cardiomyocytes, cells grown on glass coverslips were fixed in 70% ethanol, double-stained with anti-p27 and anti-sarcomeric actin antibodies, and visualized using fluorescent tyramide signal amplification of CDK4 inhibitors, p27 and p21. As substrates,FLAG-p27 or -Skp2 were prepared using 1 μg of pcDNA3Flag-p27 or pcDNA3Flag-skp2 (23) and 25 μl of rabbit reticulocyte lysate (TNT kit from Promega) according to its protocol. The in vitro p27 ubiquitylation activity was also assayed after immunoprecipitation of cell extracts with 0.4 μg of anti-Skp2 antibody.

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RESULTS

CDK2 Activity Was Suppressed and the Cell Cycle Inhibitor p27 Accumulated in the Nuclei of Cardiomyocytes after Cell Cycle Entry by D1NLS/CDK4—We have previously shown that D1NLS/CDK4 expression allowed the cardiomyocytes to enter the cell cycle (10). However, the proliferation of these cells was limited to approximately one or two cycles. To understand the mechanism by which the cells cease to proliferate, we first assayed the activity of G1 cyclin kinase, CDK4 and CDK2. As shown in Fig. 1A, the CDK4 activity was up-regulated at 24 h post-infection of D1NLS/CDK4, and remained active for at least 96 h. The CDK2 activity was also activated at 24 h, but suppressed again at 48 h, whereas the amount of CDK2 was not significantly affected. We, therefore, examined the expression of CDK2 inhibitors, p27 and p21. Fig 1B showed that p27 was accumulated in the serum-starved non-proliferating cardiomyocytes. When these cells were allowed to enter the cell cycle by D1NLS/CDK4, the p27 protein was rapidly degraded within 24 h. However, it accumulated again at 48 h, at which time CDK2 was inhibited (Fig. 1A). In REF52 fibroblasts, p27 was also rapidly degraded after entering the cell cycle by D1NLS/CDK4, but no accumulation was observed for at least 72 h. The expression of p21 was significantly suppressed in serum-starved cardiomyocytes and REF52 cells, but up-regulated at 48 h after the cell cycle entry. In Fig. 1C, immunoprecipitation of the cardiomyocyte extract was performed using anti-CDK2 antibody. Results showed that p27 was little associated with CDK2 in the serum-starved cells, but the higher amount of p27 was detected in the CDK2 complex at 48–96 h. In contrast, appreciable amounts of p21 were not detected in the CDK2 complex. It was further noted that the expression of p57 and p53, other cell cycle inhibitors, was not significantly altered in D1NLS/CDK4 cells of this study (data not shown).

Next, the immunocytological study of p27 expression was performed. As shown in Fig. 2, p27 accumulated significantly in the nuclei of D1NLS/CDK4 cells, whereas its expression was little detected in control cells. These data altogether indicated that the p27 protein was degraded after initiation of cell cycle, but was accumulated again in the nuclei during cell cycle progression of cardiomyocytes.

Serum-induced Degradation of p27 in Cardiomyocytes Was Not Different from REF52 Cells—The protein level of p27 is not significantly altered in D1NLS/CDK4 cells of this study (data not shown).

FIG. 5. Down-regulation of Skp2 was inversely correlated to the in vitro Skp2 ubiquitylation activity in cardiomyocytes. A, cardiomyocytes and REF52 cells were treated with serum (10% FBS) or D1NLS/CDK4 for 48 h, and their cell extracts (20 μg of protein) were assayed for Skp2 protein by Western blot analysis. B, cardiomyocytes and REF52 cells were treated as in A, and their total RNA (10 μg) was analyzed for Skp2 mRNA by Northern blot. C, cells were treated as in A in the presence or absence of 20 μM lactacystein, and their cell extracts were assayed for Skp2 protein by Western blot analysis. Lower panels show the Coomassie Brilliant Blue (CBB) staining as control for protein loading, D, cell extracts (50 μg of protein) were assayed for the in vitro Skp2 ubiquitylation activity as described under “Experimental Procedures.” IB, immunoblot.
apparently similar kinetics (Fig. 3B). This strongly indicates that there is no apparent difference of p27 degradation between
the cardiomyocytes and proliferating REF52 cells during their
transition from G0 to G1 phase, although cardiac cells are not
allowed to enter the cell cycle by serum treatment.

Skp2-Dependent Degradation of p27 Was Suppressed in Car-
diomyocytes—We next examined the p27 expression of cardio-
myocytes after their entry into the cell cycle by D1NLS/CDK4
expression. The p27 mRNA level in cardiomyocytes was com-
parable with that of REF52 cells during their transition from G0 to G1 phase, although cardiac cells are not
allowed to enter the cell cycle by serum treatment.

Skp2 expression caused a specific degradation of p27 in nuclei
of D1NLS/CDK4 cardiomyocytes. A, cardiomyocytes were infected with a com-

bination of AdD1NLS/AdCDK4 and an in-
creasing multiplicity of infection of Ad-
Skp2. At 48 h post-infection, cell extracts
(20 μg of protein) were prepared and as-
sayed for various cell cycle regulators by
Western blot analysis. B, total RNA (10
μg) was assayed for p27 mRNA as de-
scribed in the legend to Fig. 3A. C, car-
diomyocytes were treated as in A in the
presence or absence of 20 μM lactacystin,
and their extract (20 μg of protein) was
assayed for p27 protein. Proliferating cell
nuclear antigen (PCNA) was shown as a
loading control. D, D1NLS/CDK4 and
D1NLS/CDK4/Skp2 cardiomyocytes with
or without 20 μM lactacystin treatment
were immunostained for p27 with anti-
p27 antibody. Sarcomeric actin and cell
nuclei were also stained with anti-sarco-
meric actin antibody and 4′,6-diamidino-
2-phenylindole (DAPI), respectively (green,
p27; red, sarcomeric-actin; white, DAPI).

panel). From these data, it is indicated that cardiomyocytes
have much lower activity of Skp2-dependent p27 degradation
than the growing REF52 cells.

Skp2 Protein Was Down-regulated through Its Accelerated
Degradation in Cardiomyocytes—The data above strongly sug-
gested that the impaired degradation of p27 by Skp2 might
cause p27 accumulation during cell cycle progression of car-
diomyocytes. We, therefore, examined the expression of Skp2 in
cardiomyocytes. As shown in Fig. 5A, Skp2 protein was de-
tected in the serum-starved REF52 cells, and it was induced
after treatment with serum or D1NLS/CDK4. In cardiomyo-
cytes, however, Skp2 expression was hardly detected or signif-
cantly suppressed in the serum-starved cardiac cells, and no
significant induction was observed by serum or D1NLS/CDK4.
Northern blot analysis revealed that apparently similar
amounts of Skp2 mRNA was expressed in both cell types under
these conditions (Fig. 5B). When these cardiomyocytes were
treated with lactacystin, a proteasome inhibitor, Skp2 protein
was remarkably stabilized (Fig. 5C), indicating that Skp2 pro-
tein was expressed but actively degraded in cardiomyocytes.
Therefore, we further measured the in vitro ubiquitylation
activity of Skp2 protein. Fig. 5D revealed that Skp2 protein
was actively ubiquitylated and migrated as the multiubiquity-
lated products when assayed using extracts from cardiomyo-

The Role of p27 in Proliferating D1NLS/CDK4 Cardiomyocytes

FIG. 6.
The Role of p27 in Proliferating D1NLS/CDK4 Cardiomyocytes

We further addressed Overexpression of Skp2 Caused Specific Degradation of p27—We next examined whether the down-regulation of Skp2 protein is functionally linked to accumulation of p27 in D1NLS/CDK4 cardiomyocytes. For this purpose, the Skp2 protein was overexpressed using the adenovirus-mediated gene transfer. As shown in Fig. 6A, the expression of Skp2 caused the significant degradation of p27 in D1NLS/CDK4 cells. In contrast, p21, p57, and cyclin D1 and E were only marginally affected. The p27 mRNA level was only slightly induced by Skp2 expression (Fig. 6B). Furthermore, the decrease of p27 by Skp2 expression was abrogated by lactacystin treatment (Fig. 6C), indicating that down-regulation of the p27 protein was specifically mediated by the Skp2-dependent degradation. Immunocytochemical study revealed that the accumulation of the p27 protein in the nuclei of D1NLS/CDK4 cardiomyocytes was significantly suppressed by Skp2 expression, and this suppression was inhibited by lactacystin treatment (Fig. 6D). These data altogether indicated that Skp2 enhanced the specific degradation of p27 in the nuclei of D1NLS/CDK4 cardiomyocytes through a proteasome-dependent pathway.

Down-regulation of p27 by siRNA or Skp2 Stimulated the CDK2 Activity and Overrode the Limited Cell Cycle Progression of D1NLS/CDK4 Cardiomyocytes—We further addressed whether the accumulation of p27 had a causal effect on the early cell cycle arrest of D1NLS/CDK4-infected cardiomyocytes. For this purpose, we employed a loss-of-function approach by an RNA interference-mediated gene knockdown. As shown in Fig. 7A, three vectors expressing different p27 siRNAs specifically caused the knockdown of p27 protein in both control and D1NLS/CDK4 cells, whereas they showed no inhibition of another CDK inhibitor p21. Next, we measured the p27 protein and CDK2 activity in D1NLS/CDK4 cells expressing these siRNAs. Fig. 7B showed that the suppression of CDK2 activity was totally abrogated when the re-accumulation of p27 was knocked down by p27 siRNA. By contrast, in cells expressing control siRNA, p27 was re-accumulated and CDK2 activity was suppressed as in D1NLS/CDK4 cells (compare with Fig. 1). The p27 degradation by Skp2 also prevented the suppression of CDK2 activity in D1NLS/CDK4 cells (Fig. 7C). These data strongly indicated that p27 played a major role in suppressing CDK2 activity in D1NLS/CDK4 cells.

Based on these results, we next measured cell cycle progression and proliferation by p27 siRNA or Skp2. Fig. 8A showed that a higher proportion of the D1NLS/CDK4/p27 siRNA or D1NLS/CDK4/Skp2 cells progressed to S/G2M phase much faster than the D1NLS/CDK4 cells. Furthermore, these cells still retained the higher proportion of S/G2M cells at 192 h post-infection, compared with control D1NLS/CDK4 cells. When the cell number was counted, it was shown that p27 siRNA or Skp2 further promoted the proliferation of D1NLS/CDK4 cells (Fig. 8B). The apparent doubling time of cell proliferation was 64, 63.6, and 84 h for the D1NLS/CDK4/p27 siRNA, D1NLS/CDK4/Skp2, and D1NLS/CDK4 cells, respectively. By contrast, p27 siRNA or Skp2 expression alone had no significant effect. Therefore, it is indicated that down-regulation of p27 enhanced further stimulation of CDK2 activity and subsequent cell cycle progression of D1NLS/CDK4 cells.

Discussion

In the present study, we demonstrated that the accumulation of CDK inhibitor p27 was a strong barrier of cell cycle in terminally differentiated cardiomyocytes, and this was caused, at least in part, by the impaired Skp2-dependent p27 degradation in the nuclei of cells.

p27 is degraded at least in two phases of the cell cycle. When cells transit from the G1 to G2 phase in response to growth stimuli such as serum, p27 is degraded in the cytoplasm by the Skp2-independent pathway. On the other hand, in proliferating cells, p27 is degraded in the nuclei during S and G2 phase by a Skp2-dependent pathway (17, 19). The impaired Skp2-dependent nuclear degradation of p27 in cardiomyocytes is sup-

FIG. 7. Specific down-regulation of p27 by siRNA or Skp2 abrogated early suppression of CDK2 activity in D1NLS/CDK4 cells. A, cardiomyocytes were untreated or infected with a combination of AdD1NLS/AdCDK4 and adenovirus vectors encoding p27 siRNA 1, 4, 6, or hepatitis C viral protein (control). At 48 h post-infection, cell extracts (20 μg of protein) were prepared and assayed for p27, p21, and cyclin D1 by Western blot analysis. Sarcomeric actin was shown as control for protein loading. B, cells were infected with a combination of AdD1NLS/AdCDK4 and virus vector for control siRNA or p27 siRNA (#6). At the indicated time, cell extracts (20 μg) were assayed for p27 and CDK2 kinase (Histone H1 kinase) activity as described under “Experimental Procedures.” C, cells were infected with a combination of AdD1NLS/AdCDK4 and an increasing multiplicity of infection of AdSkp2, and after 48 h, CDK2 activity was assayed as in B.
ported by the following findings, (i) *in vitro* p27 ubiquitylation activity of cell extracts or the Skp2 immunocomplex was remarkably suppressed in cardiomyocytes compared with proliferating REF52 fibroblasts (Fig. 4, B and C); (ii) p27 accumulation was observed in the nuclei of D1NLS cells (Fig. 2); (iii) p27 in serum-starved cardiomyocytes and REF52 cells exhibited apparently similar kinetics of degradation after serum stimulation (Fig. 3), suggesting no apparent difference of the Skp2-independent degradation between these cells. (iv) Skp2 protein was actively ubiquitylated in cardiomyocytes compared with REF52 cells (Fig. 5D). Thus, it is strongly argued that increased Skp2 degradation is one of the mechanisms by which cardiomyocytes accumulated p27 in the nuclei and ceased to grow early after the forced cell cycle progression by nuclear cyclin D1.

Other cell cycle inhibitors, p21 and p57, are also recognized and degraded by the Skp2-dependent pathway (25, 26). After cell cycle entry of cardiac cells as well as REF52 cells, p21 was also up-regulated (Fig. 1B). However, it was not significantly associated with CDK2 (Fig. 1C). Furthermore, p21 degradation in Skp2-expressed cells was less than p27, and p57 was neither induced nor affected significantly by Skp2 expression (Fig. 6A). Thus, we speculate it is unlikely that these inhibitors play a major role in cell cycle arrest of D1NLS cardiomyocytes. Inconsistent with this, the knockdown of p27 using the siRNA approach promoted the proliferation of D1NLS/CDK4 cells, indicating that p27 rather than p21 or p57 played a role in causing cell cycle arrest. It is interesting to note here that p27 is highly accumulated with concomitant reduction of cell growth in Skp2-deficient cells (23). In these mice, however, cardiac size or cell number was not significantly affected. In contrast, p27 knockout mice show the enlarged heart with much higher proliferative activity (21). These are consistent with our findings that Skp2 and p27 are reciprocally regulated and the specific p27 down-regulation by siRNA or Skp2 enhanced the proliferation of D1NLS/CDK4 cells. The role of cyclin kinase inhibitors p27, p21, or p57 in suppressing cell cycle progression might depend on the cell type and cellular context. Indeed, it is reported that the expression of these inhibitors in tissue is not uniform (27–29). Thus, more investigation might reveal the functional implication of these inhibitors in cell cycle arrest of terminally differentiated cells.

Cyclin E, which is a component of the late G1/cyclin kinase CDK2 and is also recognized by Skp2 (23, 30), appeared to be regulated in a biphasic manner. It was up-regulated at low expression of Skp2, but down-regulated at a higher level of Skp2 (Fig. 6A). The up-regulation of cyclin E might support cell cycle progression of D1NLS/CDK4/Skp2 cells, whereas its down-regulation at the higher Skp2 level might cause another barrier for cell cycle progression. This is also an another issue of future study.

The heart is developed at the early stage of embryogenesis during which cardiomyocytes retain the ability to proliferate, but they lose the capacity to proliferate in terminal differentiation. Our present study raises an interesting possibility that the down-regulation of Skp2 by increased degradation might be

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**Fig. 8.** p27 down-regulation by siRNA or Skp2 promoted proliferation of D1NLS/CDK4 cardiomyocytes. A, cells were infected with a combination of AdD1NLS/AdCDK4 along with Adp27 siRNA (6) or AdSkp2. At each time indicated, cells were harvested and subjected to laser scanning cytometer analysis as described under “Experimental Procedures.” B, cardiomyocytes were untreated or infected with a combination of AdD1NLS, AdCDK4, Adp27siRNA (6), or AdSkp2 as indicated and cultured. Cell number was counted on the indicated day after infection, and the relative cell proliferation was expressed as mean ± S.E. of three independent experiments.
causally related to the loss of proliferation of cardiomyocytes during terminal differentiation. Inconsistent with this, we observed that fetal rat cardiomyocytes, which decrease cell growth capacity but are still capable of proliferating, exhibited the intermediate activity of the in vitro Skp2 ubiquitylation and p27 expression between neonatal cardiac cells and REF52 fibroblasts. More recently, it has been reported that Skp2 and its cofactor Cks1 proteins are degraded by the ubiquitin ligase APC/Cdcl1 (anaphase-promoting complex/cyclosome and its activator Cdh) (31, 32). Detailed analysis of APC expression and its Skp2 degrading activity in cardiomyocytes may clarify the mechanism of terminal differentiation and such an investigation is now in progress.

Finally, we demonstrated that p27 is a strong cell cycle barrier of terminally differentiated cardiomyocytes, and its down-regulation by a combination of p27 siRNA or Skp2 and D1NLS/CDK4 significantly overrode the limited cell proliferation of cardiac cells. The impaired Skp2/p27 regulation may be causally related to the loss of proliferation of cardiomyocytes and such an investigation is now in progress.

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