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Signal induced ubiquitination of p57Kip2 is independent of the C-terminal consensus Cdk phosphorylation site

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Abstract **The cyclin-dependent kinase inhibitor $p57^{Kip2}$ is required for normal mouse embryonic development. $p57^{Kip2}$ consists of four structurally distinct domains in which the conserved C-terminal nuclear targeting domain contains a putative Cdk phosphorylation site (Thr^{342}) that shares a great similitude in the adjacent sequences with $p27^{Kip1}$ but not with $p21^{Cip1}$. Phosphorylation on Thr^{187} has been shown to promote degradation of $p27^{Kip1}$. Although there is sequence homology between the C-terminal part of $p27^{Kip1}$ and $p57^{Kip2}$, we show that the ubiquitination and degradation of $p57^{Kip2}$ are independent of Thr^{342}. In contrast a destabilizing element located in the N-terminal is implicated in $p57^{Kip2}$ destabilization.**

Key words: $p57^{Kip2}$; T342A; Phosphorylation; Ubiquitination

1. Introduction

Proliferation is positively regulated by cyclin-dependent kinases (Cdks), a family of highly regulated enzymes that link proliferative signals with mechanical aspects of cell duplication. In opposition to Cdks are Cdk inhibitors (Ckis) that induce cell cycle arrest in response to anti-proliferative signals, including contact inhibition, serum deprivation [1] and cell differentiation [2–5]. Ckis can be divided in two families. The Ink4 family includes $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{ARF}$. These proteins specifically bind and inhibit Cdk4 and Cdk6 and not other Cdks such as Cdk2. $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, members of the other family of inhibitors, the Cip/Kip family, have the ability to inhibit all G1/S phase cyclin–Cdk complexes [6]. However, of the Ckis, only $p57^{Kip2}$ is required for embryonic development [7]. Loss of $p57^{Kip2}$ results in proliferative disorders and defects in development of several tissues [8]. The amounts of Ckis expressed in cells may be crucial in controlling cell cycle progression [9], cell differentiation [10] and terminally differentiated cells in mouse embryo [11]. Expression of either $p57^{Kip2}$ or $p21^{Cip1}$ is a requirement for muscle differentiation as shown by double knockout of $p57^{Kip2}$ and $p21^{Cip1}$ that gave the same muscle-deficient phenotype as observed with myogenin (−/−) mice [12]. Up-regulation of $p57^{Kip2}$ has been shown to stabilize MyoD by blocking cyclin E–Cdk2 activity [10] and by direct interaction with MyoD [13] suggesting that a fine balance exists between cell cycle regulation and terminal differentiation.

The concentration of $p27^{Kip1}$ is though to be regulated predominantly by posttranslational mechanisms [14,15]. $p27^{Kip1}$ is degraded by both ubiquitin-independent proteolytic cleavage and the ubiquitin–proteasome pathway [16]. Regulation of ubiquitin-mediated proteolysis is often achieved by phosphorylation of the target protein, which renders it more susceptible to degradation. $p27^{Kip1}$ down-regulation is promoted by its phosphorylation on Thr^{187} by the cyclin E–Cdk2 complex and by various kinases such as mitogen-activated protein kinases [17,18]. Recent data have shown that Skp2, an F-box protein that functions as the receptor component of an SCF (Skp1/Cul1/F-box protein) ubiquitin ligase complex, binds to and ubiquitinates $p27^{Kip1}$ only when Thr^{187} is phosphorylated [19,20]. These observations indicate that phosphorylation of $p27^{Kip1}$ on Thr^{187} controls its stability. Because much less is known about the role and the regulation of $p57^{Kip2}$, in the present report we have examined whether or not Thr^{342} in $p57^{Kip2}$ that is equivalent to Thr^{187} in $p27^{Kip1}$ is responsible for the accelerated degradation of $p57^{Kip2}$. We show that Thr^{342} is not a major phosphorylation site of $p57^{Kip2}$ in muscle cells and is not implicated in the ubiquitination and the degradation of $p57^{Kip2}$. Further analysis suggests that a destabilizing domain in the NH_2-terminal part of $p57^{Kip2}$ is implicated for its degradation.

2. Materials and methods

2.1. Cell cultures and DNA transfection

The mouse skeletal muscle cell line C2C12 was maintained in growth medium supplemented with antibiotics and 20% fetal calf serum in Dulbecco's modified Eagle's medium. C2C12 cells were transfected by the calcium phosphate procedure as described previously [9].

2.2. Plasmids and cycloheximide treatment

For Gal4 fusion experiments, we first created an expression vector pCMV-Gal4-HA by inserting in frame the Hind*III*–Cla*I* filled in fragment of Gal4 DNA binding domain at the Eco*RV* site of pCMV-3HA. $Gal4_{DBD}$ fusion proteins were generated by PCR amplifying cDNA encoding various domains of $p57^{Kip2}$ and ligating in frame the PCR products into pCMV-Gal4-HA. C2C12 myoblasts were transfected with pCDNA3-HA-$p57^{Kip2}$, pCDNA3-HA-$p57^{Kip2}$ T342A or the various mutants in six-well plates [10]. Transfected cells were treated with cycloheximide (Sigma) at 50 μg/ml for the indicated times and harvested for Western blot analyses and $p57^{Kip2}$ proteins were detected by immunoblotting [10]. α-Tubulin was used as an internal control. Western blot were scanned and quantified by using a GelScan (Pharmacia).

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2.3. Antibodies, immunoprecipitation and Western blot analyses

For immunoprecipitation, whole cell extracts were prepared in immunoprecipitation buffer [10]. Precleared cell lysates were incubated with the indicated antibodies for 2–3 h at 4°C with gentle agitation. Immunocomplexes bound to protein G-Sepharose were then washed several times in immunoprecipitation buffer. Immunoprecipitated proteins were resolved by 10% SDS–PAGE. Electrophoretic transfer of proteins from SDS–PAGE gels to nitrocellulose membranes was blocked with Tris–HCl 50 mM, pH 7.4, 150 mM NaCl and 0.05% Tween 20 containing 5% skimmed milk and incubated overnight at 4°C with primary antibodies. Polyclonal (C20) anti-p57^{Kip2} was provided by Santa Cruz (Biotechnology, Santa Cruz, CA, USA). The monoclonal 12CA5 anti-HA antibody was provided by Boehringer (Mannheim, Germany) and anti-α-tubulin (clone DM1A) was supplied by Sigma. Membranes were washed and incubated for 1 h with a peroxidase-conjugated secondary antibody (Sigma). Membranes were incubated with an enhanced chemiluminescence system (ECL, Amersham) according to the manufacturer's instructions.

2.4. Immunofluorescence staining

Cells were cultured on coverslips and fixed in 2% paraformaldehyde for 30 min at 4°C, and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. The cells were immunostained with anti-HA antibodies (12CA5). The Texas red-conjugated F(ab/)$_2$ fragment of donkey anti-mouse IgG was used to visualize the mouse monoclonal antibodies.

3. Results

3.1. Phosphorylation of p57^{Kip2} in vivo

The C-terminal domain of mouse and human p57^{Kip2} contains a consensus Cdk phosphorylation site conserved in p27^{Kip1} together with adjacent sequences (Fig. 1A). We focused on the potential role of this site in determining the stability of p57^{Kip2} because phosphorylation of p27^{Kip1} on Thr^{187} by Cdk2 is though to initiate the major pathway for p27^{Kip1} proteolysis [19,20]. We generated a mammalian expression vector encoding a mutant mouse p57^{Kip2} protein in which Thr^{342} was replaced with Ala (T342A). The phosphorylation status of p57^{Kip2} T342A was investigated by transiently expressing the HA-epitope-tagged wild-type and mutant proteins in C2C12 cells and metabolically labeling the cells with ^{32}Pi. p57^{Kip2} proteins were then immunoprecipitated with anti-HA and the extend of ^{32}P incorporation was evaluated by autoradiography and image analysis and normalized by the amount of p57^{Kip2} protein estimated by immunoblot analysis of the immunoprecipitates with anti-p57^{Kip2} (Fig. 1B). The amount of ^{32}P incorporated by the T342A mutant was virtually identical to that incorporated by wild-type protein in C2C12 muscle cells. These results indicated that Thr^{342} is not a major phosphorylation site in p57^{Kip2}. Anti-HA immunostaining of culture expressing HA-p57^{Kip2} wt and/or p57^{Kip2} T342A showed identical nuclear localization of the two proteins (Fig. 1C). Characterization of p57^{Kip2} levels in the presence of cycloheximide revealed that mutation T342A

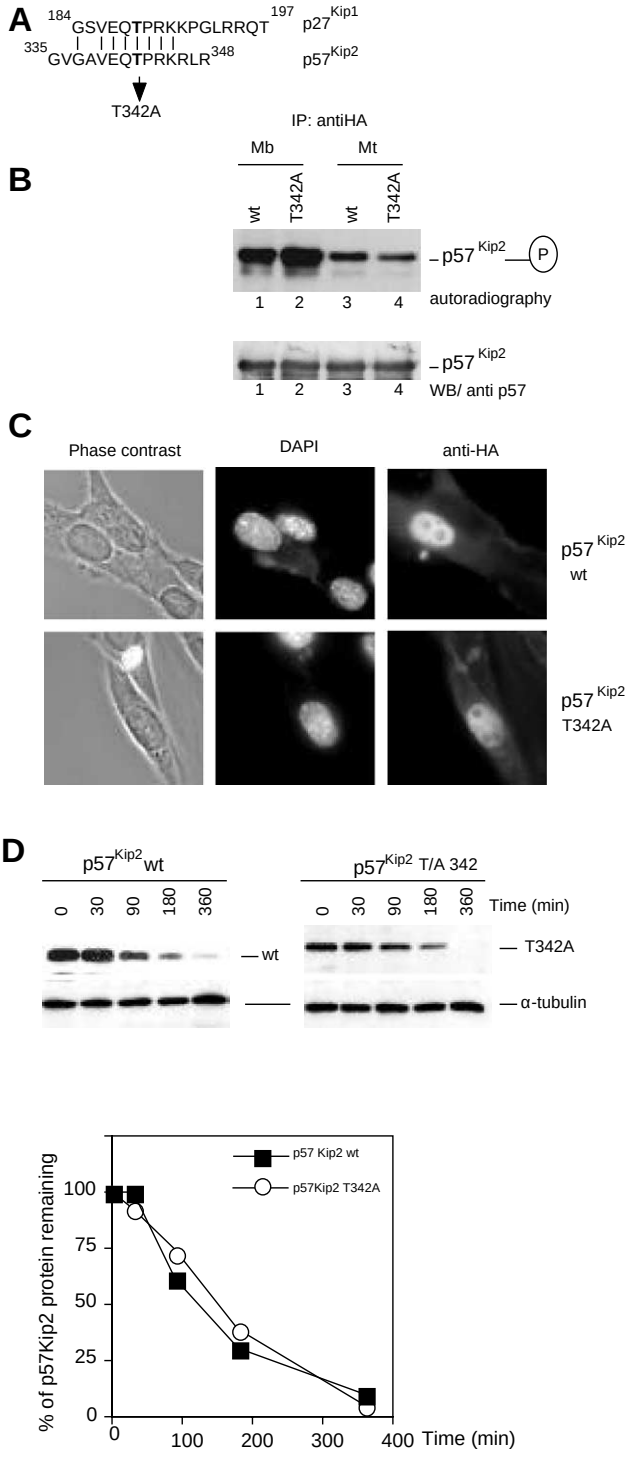

Fig. 1. A: Alignment of the putative C-terminal Cdk phosphorylation site in mouse p27^{Kip1} and p57^{Kip2}. Mutation of threonine residue in alanine is in bold (T342A). B: C2C12 myoblasts (Mb) were transfected with expression vectors encoding HA-p57^{Kip2} wild-type (wt) or HA-p57^{Kip2} T342A. Forty-eight hours after transfection duplicate transfected cells were transferred in differentiation medium for 3 days (Mt). Mb and Mt were metabolically labeled with [^{32}P]orthophosphate for 2 h. Cells were collected and total cell lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates were transferred onto nitrocellulose membranes. Shown is an autoradiogram of the radioactivity incorporated into p57^{Kip2} and a Western blot analysis of the same membrane probed with the anti-p57^{Kip2} polyclonal antibody. C: C2C12 myoblasts were transfected with mammalian expression plasmids encoding HA-p57^{Kip2} wt or HA-p57^{Kip2} T342A. Twenty-four hours later, the transfected cells were fixed and immunostained with anti-HA antibody and DAPI and examined by indirect immunofluorescence microscopy. D: After addition of cycloheximide to the culture medium, transfected cells were harvested at various times and processed for Western blot analysis of p57^{Kip2} proteins by using anti-HA antibody. The decay of p57^{Kip2} proteins is depicted in the graph (lower panel).

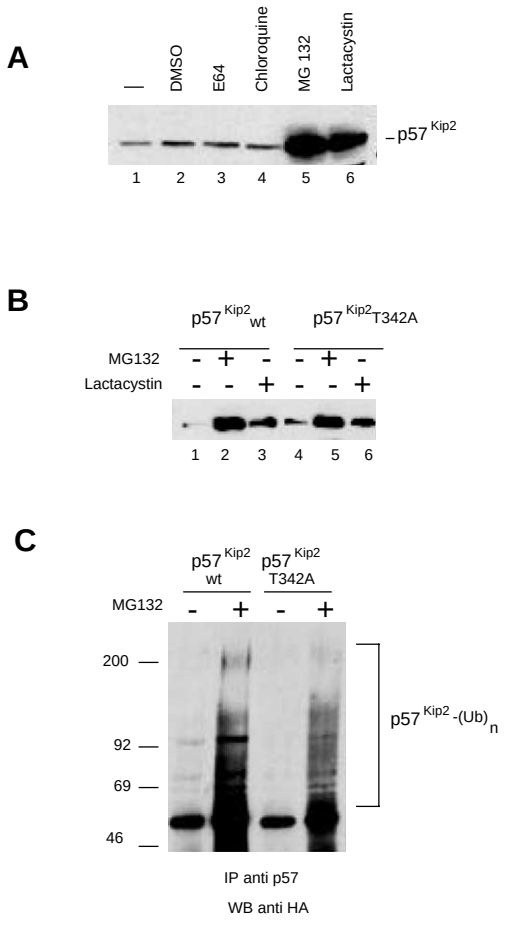

Fig. 2. Inhibition of the proteasome activity induces $p57^{Kip2}$ protein accumulation. A: C2C12 myoblasts were transiently transfected by an expression vector encoding HA-$p57^{Kip2}$ wt. Forty-eight hours later, the level of $p57^{Kip2}$ was monitored in the absence (lane 1) or in the presence of DMSO (lane 2), the lysosomal cysteine protease inhibitor E64 (30 μM, lane 3), the general inhibitor of lysosomal proteolysis chloroquine (100 μM, lane 4) or the proteasome inhibitors lactacystin (10 μM, lane 5) and MG132 (50 μM, lane 6). Cell extracts were prepared and $p57^{Kip2}$ accumulation was analyzed by Western blotting. B: C2C12 myoblasts were transiently transfected with expression vectors encoding $p57^{Kip2}$ wt or $p57^{Kip2}$ T342A, treated with MG132 or lactacystin and processed as in panel A. C: C2C12 myoblasts were transiently cotransfected with expression plasmids encoding HA-ubiquitin, HA-$p57^{kip2}$ wt or the mutant T342A. Forty-eight hours later, transfected cells were treated for 4 h with 20 μM MG132 (+) or not (−) before harvesting. Cells were lysed in denaturation buffer containing 1% SDS, boiled for 5 min at 95°C. The lysates were then diluted in immunoprecipitation buffer, subjected to immunoprecipitation with anti-$p57^{Kip2}$ antibody, and analyzed by Western blotting with anti-HA antibodies.

did not significantly modify protein stability (Fig. 1D). Altogether these data indicate that $p57^{Kip2}$ T342A remained unaffected by mutation of the putative Cdk phosphorylation site.

3.2. *$p57^{Kip2}$ T342A is ubiquitinated and degraded by the proteasome in vivo*

We investigated the effect of different protease inhibitors on the amount of $p57^{Kip2}$ wt in asynchronous C2C12 myoblasts. Addition of MG132 or lactacystin, two specific inhibitors of the 26S proteasome, induced an accumulation of $p57^{Kip2}$ wt protein after 4 h of treatment (Fig. 2A). In contrast, the cysteine protease inhibitor E64 and/or chloroquine, an inhibitor of lysosomal proteolysis, both were ineffective, showing that the proteasome pathway is mainly involved in the degradation of $p57^{Kip2}$. We next compared the effects of MG132 and lactacystin on the mutant $p57^{Kip2}$ T342A protein level. Addition of MG132 (Fig. 2B, lanes 2 and 5) or lactacystin (lanes 3 and 6) but not DMSO (lanes 1 and 4) induced an accumulation of $p57^{Kip2}$ T342A identical to the wild-type protein. Altogether, these data showed that in myoblasts the proteasome pathway degrades $p57^{Kip2}$ independently of Thr^{342}. Ubiquitination is known to have important roles in the control of the proteasome degradation pathway. Therefore we tested whether $p57^{Kip2}$ wt as well as mutant T342A could also be modified by ubiquitination. Expression vectors encoding the wild-type and the mutant T342A were cotransfected with a plasmid encoding HA-tagged ubiquitin. Twenty-four hours after transfection, C2C12 myoblasts were treated or not with MG132 for 4 h. $p57^{Kip2}$ proteins were immunoprecipitated with anti-$p57^{Kip2}$ antibody from denatured cell lysates and immunoprobed with anti-HA to detect immunoprecipitated as well as ubiquitinated $p57^{Kip2}$ proteins. In the presence of MG132, both wild-type and the mutant T342A showed a high degree of ubiquitination confirming that Thr^{342} is not implicated in the ubiquitination and the degradation of $p57^{Kip2}$ (Fig. 2C).

3.3. *The NH_2 domain of $p57^{Kip2}$ is required for rapid proteolysis*

We next asked which region of $p57^{Kip2}$ is required for its proteolysis. To do this, we constructed various $p57^{Kip2}$ deletion mutants either cloned in frame with the $Gal4_{DBD}$ (Fig. 3A) or not (Fig. 3B). We transiently expressed these deletion mutants in C2C12 myoblasts and measured their stability. Deletion of the amino acids 1–105 (ΔN1) greatly stabilized $p57^{Kip2}$ by increasing its half-life from 3 h up to 6 h. Deletion of the COOH domain (ΔC1) did not affect $p57^{Kip2}$ stability and deletion of the central part plus the COOH domain of

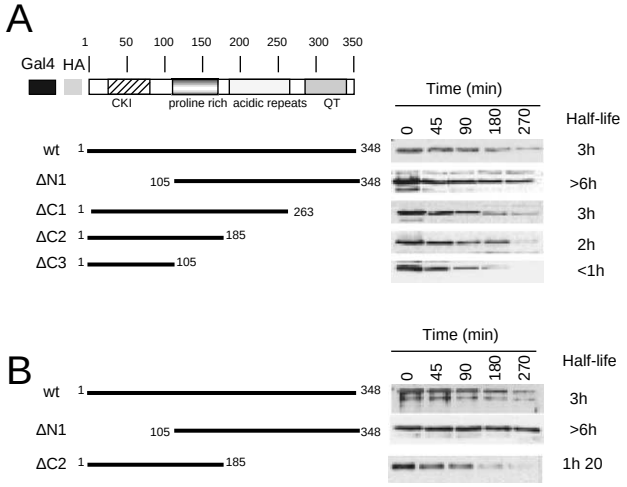

Fig. 3. The NH_2-terminal domain is implicated in the destabilization of $p57^{Kip2}$. A: Schematic diagram of the $p57^{Kip2}$ deletion mutants indicating the main regions of the protein, which is fused to $Gal4_{DBD}$ (left panel). C2C12 myoblasts were transfected with each indicated $Gal4_{DBD}$-HA-tagged $p57^{Kip2}$ mutant and cells were then treated as in Fig. 1D. B: Expression vectors encoding HA-tagged $p57^{Kip2}$ constructs were transiently transfected in C2C12 myoblasts and treated as in Fig. 1D.

$p57^{Kip2}$ (ΔC2) reduced the half-life to ~2 h. On the other hand deletion of the amino acids 106 to 348 (ΔC3) greatly destabilized $Gal4_{DBD}$-$p57^{Kip2}$, by decreasing its half-life to less than 1 h suggesting that the amino acids 1–105 contain a destabilizing element. These data were confirmed in Fig. 3B. This domain in $p57^{Kip2}$ has been shown to contain the cyclin and Cdk binding sites implicated in the inhibition of cyclin–Cdk complexes [1].

4. Discussion

The phosphorylation state of many proteins affects their stability and phosphorylation of $p27^{Kip1}$ on Thr^{187} is a central mechanism in the control of the stability of $p27^{Kip1}$ by ubiquitin-mediated degradation [14–20]. Because Thr^{187} in $p27^{Kip1}$ and Thr^{342} in $p57^{Kip2}$ are located in a C-terminal conserved domain (Fig. 1A), the potential role of Thr^{342} on $p57^{Kip2}$ phosphorylation and stability was studied. Our results show that $p57^{Kip2}$ is phosphorylated in vivo and is mainly degraded by the proteasome pathway. Mutation of Thr^{342} in a non-phosphorylatable alanine residue (T342A) does not modify the phosphorylation state nor the stability of $p57^{Kip2}$ in C2C12 muscle cells. Our data demonstrate that ubiquitination and degradation of $p57^{Kip2}$ are independent of Thr^{342} leading us to the hypothesis that another posttranslational modification and/or domain in $p57^{Kip2}$ is implicated in its destabilization. This finding is strengthened by the fact that $p57^{Kip2}$ does not physically interact with Skp2 (data not shown), the F-box protein component of the SCF^{Skp2} implicated in the ubiquitination and the degradation of $p27^{Kip1}$ phosphorylated on Thr^{187} [19,20]. Posttranslational modifications of target proteins are implicated in the recognition by certain SCF-type E3 ubiquitin ligases and subsequent degradation by the 26S proteasome. These include phosphorylation [21], proline hydroxylation [22], *N*-glycosylation [23]. On the other hand, ubiquitination of substrates requires direct binding of particular domain(s) between the F-box protein and the substrate [24]. By using deletion and substitution mutants, we identified a destabilizing domain in $p57^{Kip2}$ protein located between amino acids 1 and 105. This NH_2 region contains three conserved domains: the cyclin and Cdk binding sites and the 3-_{10} helix motif. This latter has been shown to be implicated in the basis of a molecular mechanism by which $p57^{Kip2}$ but not $p21^{Cip1}$ nor $p27^{Kip1}$ inhibits Cdk2 kinase activity [25]. This conserved domain in human and mouse $p57^{Kip2}$ suggests that it may play an important role in the substrate recognition. Also it may be possible to use the powerful genetic approaches available for yeast to identify the components involved in the $p57^{Kip2}$ targeting and proteolysis systems, which regulate $p57^{Kip2}$ levels.

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