Serine 123 Phosphorylation Modulates p21 Protein Stability and Activity by Suppressing Ubiquitin-independent Proteasomal Degradation*

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Received for publication, May 24, 2012, and in revised form, August 17, 2012. Published, JBC Papers in Press, August 20, 2012, DOI 10.1074/jbc.M112.384990

The cyclin-dependent kinase inhibitor p21Waf1/Cip1 is a major regulator of the cell cycle and plays an important role in many cellular processes, including differentiation, stress response, apoptosis, and tumorigenesis. We previously cloned the gene encoding dog p21 and found that unlike its human ortholog, dog p21 is expressed as two isoforms, one high molecular mass band of 23 kDa and one low molecular mass band of 19 kDa. In the current study, we found that the high molecular mass band is phosphorylated, whereas the low molecular mass band is hypophosphorylated. Moreover, by generating multiple mutants of dog p21, we found that serine 123 and proline 124, which form a consensus site for proline-directed phosphorylation, are required for expression of the high molecular mass p21 isoform through phosphorylation at serine 123. Most importantly, we showed that serine 123 phosphorylation inhibits ubiquitin-independent proteasomal degradation of p21 protein and subsequently, prolongs p21 protein half-life and enhances the ability of p21 to suppress cell proliferation. Taken together, these data reveal that serine 123 phosphorylation modulates p21 protein stability and activity by suppressing ubiquitin-independent proteasomal degradation.

Background: p21 is a major regulator of the cell cycle.

Results: Serine 123 phosphorylation, which leads to expression of two dog p21 isoforms, modulates p21 protein stability and activity by suppressing ubiquitin-independent proteasomal degradation.

Conclusion: Serine 123 phosphorylation is critical for modulating p21 protein stability and activity.

Significance: This study provides new insight into the regulation of p21 via phosphorylation.
result of proline-directed phosphorylation at serine 123. The dog p21 with a high molecular mass is a phosphorylated form whereas the dog p21 with a low molecular mass is an underphosphorylated form. To determine the role of serine 123 phosphorylation in modulating dog p21 activity, we showed that serine 123 phosphorylation enhances the ability of dog p21 to block S phase entry and consequently to suppress cell proliferation. Furthermore, we showed that serine 123 phosphorylation prolongs the half-life of dog p21 protein, at least in part, by preventing dog p21 from the ubiquitin-independent proteasomal degradation.

MATERIALS AND METHODS

Reagents—Anti-HA was purchased from Covance (San Diego, CA). Anti-p53 (FL393) and anti-GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin, MG132, and cycloheximide were purchased from Sigma. Lipofectamine 2000 was purchased from Invitrogen.

Cell Culture and Transfection—MDCK (ATCC CCL-34), D17 (ATCC CCL-183), and Cf2Th (ATCC CRL-1430) cell lines were purchased from ATCC. These cells were maintained in DMEM (Dulbecco-Vogt modified Eagle’s MEM) supplemented with 10% fetal bovine serum and 1% nonessential amino acid. DMEM (Dulbecco-Vogt modified Eagle’s MEM) supplemented with 10% fetal bovine serum. For transfection of various p21 plasmids, Lipofectamine 2000 was used according to the manufacturer’s instructions (Invitrogen).

Cell Line Generation—Cf2Th cells that can inductively express HA-tagged wild-type dog p21, dog p21(S123A), and dog p21(S123D), were generated by using a Tet-on inducible system as described previously (33, 34). Briefly, pcDNA4 vectors containing HA-tagged wild-type dog p21, dog p21(S123A), or dog p21(S123D) were transfected into Cf2Th cells expressing a tetracycline repressor (pcDNA6). p21-expressing cells were selected with zeocin and confirmed by Western blot analysis. For induction, tetracycline (250–500 ng/ml) was added to the media and live cells on the plate were collected and fixed p21 for 3 days. After induction, both floating and dead cells in the media and live cells on the plate were counted three times using a Coulter cell counter.

Cells were then fixed in methanol/glacial acetic acid (7:1), washed with water, and stained with crystal violet (0.2 g/liter).

DNA Histogram Analysis—Cells were seeded at 2 × 10^5/90-mm-diameter plate and uninduced or induced to express dog p21 for 3 days. After induction, both floating and dead cells in the media and live cells on the plate were collected and fixed with 10 ml of 75% ethanol overnight and then resuspended in 300 μl of PBS solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACS Calibur; BD Biosciences) within 4 h. The percentage of cells in the sub-G1, G1, S, and G2/M phases was determined by the Cell Quest program.

Plasmids and Mutagenesis—pcDNA3-HA-dog p21 vector was generated as described previously (32). The various dog p21 mutants were generated using PCR-based mutagenesis and confirmed by sequencing. Specifically, dog p21 cDNA was used as a template for PCR amplification with one pair of common primers and one pair of specific primers. The PCR product was sequenced and then inserted to pcDNA3 vector through HindIII and EcoRI. The common primers for all dog p21 mutants are: upstream primer, 5’ ATC CAA GCT TCC GCC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT TCG GAG CCG TCC AGG GAC G G 3’ and downstream primer, 5’ ATC CGA ATT CAG ATT AGG CCT TCT TCG GAG 3’. The specific primers for dog p21(Hu 38–74) mutant are: upstream primer, 5’ ATG GCG GGC TGC ATC CAG GAG GCC CGT GAG GGA TGT AAC TT’ T GAC TCT 3’ and downstream primer, 5’ CTC ACG GGC CTC CTT GTG ATT CAG CAG CTC GAG GTC ACA GTC CCG 3’. The specific primers for dog p21(Hu 74–83) mutant are: upstream primer, 5’ CCA AAG CTC TAC TTT CCC ACG GGG CCC GGG GGG GGC CGT GAC GTG AGG 3’ and downstream primer, 5’ CCG GGG CCC GTG GAG GGA GAT GGA GCT GAG TTT GGG CAC GAG GTC ACA CTC CCG 3’. The specific primers for dog p21(Hu 84–93) mutant are: upstream primer, 5’ CGA GGG GGG GGC CGT GAT TGG GGA GGA GGC AGG CGG CCC GCC GCC ACC TCG CCT G 3’ and downstream primer, 5’ CCT GCC TCC TCA CAA CCT ATC ACG GCC TCG CCG GGG CCC GGC ACC AGG GAC 3’. The specific primers for dog p21(Hu 107–116) are: upstream primer, 5’ GAG GAA GAC CAT GTG GAC CTG TCA CTG TCT TGG ACC CTC GTG CCT CAC TC 3’ and downstream primer, 5’ AGA CAG TGA CAG GTC CAC ATG GTC TTC CTC AGT GTG CCC TGG CAG GAC 3’. The specific primers for dog p21(Hu 117–126) are: upstream primer, 5’ TGT ACC TTT GTG CCT GCC TCA GGG GAG CCT CAG TCT GCA TCC CCG GTG G G 3’ and downstream primer, 5’ CTC CTC CCC TGA GCG AGG CAC AAG GGT ACA GGT CAG CTA CGA GTC CAG G 3’. The specific primers for dog p21(Hu 127–136) are: upstream primer, 5’ GCT GAA GGG TCC CCA GGT GGA CCT GCA CCT GAC TGT CCT GAG CGA AAA CGG 3’ and downstream primer, 5’ GTTC TCC AGG GCC ACC TGG GGA CCC CCT ACC CTC GAG GGA GTT AGG 3’. The specific primers for dog p21(L120V) mutant are; upstream primer, 5’ TGC ACC TCT GTG CCT GCT ACC CTC CCT CC 3’ and downstream primer, 5’ GTG AGG CAC GAG GGT GCA GGT CAG C 3’. The specific primers for dog p21(H122R) mutant are; upstream primer, 5’ C CGT CCT CGC TCC CCT CAG CCG 3’ and downstream primer, 5’ CAG GGG AGC GAC GCA GGA GGG TG 3’. The specific primers for dog p21(P124G) mutant are; upstream primer, 5’ CCT CAC TCC GGG GAG CGG CCT CTC GAG GCA TC 3’ and downstream primer, 5’ AGG CCG CAC CCC GGA GTG AGG GAG 3’.
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TCC 3’ and downstream primer, 5’ TGC CTC AGG CTG CTC AGG GGA GTG AG 3’. The specific primers for dog p21(S123A) mutant are: upstream primer, 5’ CTG CCT CCC GCC CCT GAG CGG CCT GAG G 3’ and downstream primer, 5’ CGG CTC AGG GGG GTG AGG CAG GAG GGT GC 3’. The specific primers for dog p21(S123D) mutant are: upstream primer, 5’ CTG CCT CCC GAG CCT GAG CGG CCT GAG G 3’ and downstream primer, 5’ CCG CTC AGG GGA GTG AGG CAG GAG GGT GC 3’. The specific primers for human p21(117–126) mutant are: upstream primer, 5’ TGC ACC CTC CTC GCT CAT CCT GCT AAG GAG GGT GCA AGA AGA CAG TG TCA GAT GTC CAC ATG GTC 3’ and downstream primer, 5’ AGC CTG CTC AGG TGA GGG AGG CAC AAG GGT AC 3’.

To generate various human p21 mutants, the same strategy was used with human p21 cDNA as a template. The common primers for all human p21 mutants are: upstream primer, 5’ ATC CAA GCT TGC CAT GTC AGA ACC GGC TGG GGA TG 3’ and downstream primer, 5’ ATC CGA ATT CAG ATT AGG GCT TCC TCT TGG AG 3’. The specific primers for human p21(Dog117–126) mutant are: upstream primer, 5’ TGC ACC CTC CTC GCT CAT CCT GCT AAG GAG GGT GCA AGA AGA CAG TG TCA GAT GTC CAC ATG GTC 3’ and downstream primer, 5’ CCG CTC AGG GGA GTG AGG CAG GAG GGT GC 3’.

To generate human p21(117–136) mutant, a first PCR was performed using human p21 as a template with upstream primer P1, 5’ ATT CAA GCT TGC CGC CTC TAC CCA TAC GAT GTT CCA GAT TAC CCT GCT TCA GAA CCG CCT GGG GAT G 3’ and downstream primer P2, 5’ CCG CTC AGG GGA GTG AGG CAG GAG GGT GCA AGA AGA CAG TG TCA GAT GTC CAC ATG GTC 3’. Next, a second PCR was performed using dog p21 as template with upstream primer P3, 5’ TGC ACC CTC CTC CTC CCT GCT CAC TCC CCT GAG CGG CCT GAA GGG TCC CCA GGT GC 3’ and downstream primer P4, 5’ ATC CGA ATT CAG ATT AGG GCT TCC TCT TGG AG 3’. The products from these two PCR were mixed and used as a template for a third PCR using primers P1 and P4. The resulting PCR product was then sequenced and cloned into pcDNA3 through HindIII and EcoRI.

To generate pcDNA4-HA-dog p21, pcDNA4-HA-dog p21(S123A), and pcDNA3-HA-dog p21(S123D), a cDNA fragment containing HA-tagged wild-type dog p21, dog p21(S123A), and dog p21(S123D) was digested from pcDNA3-HA-dog p21, pcDNA3-HA-dog p21(S123A), and pcDNA3-HA-dog p21(S123D), respectively, and then inserted into pcDNA4 vector through HindIII and EcoRI.

Western Blot Analysis—Western blot analysis was performed as described previously (35). Briefly, cells were washed and collected from plates in PBS solution, resuspended with 2× SDS sample buffer, and boiled for 5 min. Proteins were then resolved in an 8–12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, followed by ECL detection. The level of protein was quantified using Labworks software (UVP, Upland, CA).

RESULTS

Phosphorylation Is Responsible for Expression of Two p21 Isoforms in Dog—Previously, we cloned the gene encoding dog p21, which contains 164 amino acids and shares 82% amino acid identity to that of human p21(32). Interestingly, unlike the human p21, the dog p21 is expressed as two isoforms, one 23-kDa isoform and one 19-kDa isoform (Fig. 1A, compare lane 1 with lanes 4 and 7, respectively) (32). In addition, upon treatment with a DNA-damaging agent, these dog p21 isoforms were elevated in MDCK and D17 cells, both of which carry wild-type p53 (Fig. 1A, compare lanes 4 and 7 with lanes 5–8 and 9, respectively). We would like to mention that MDCK is an immortalized but untransformed dog kidney cell line whereas D17 is a dog osteosarcoma cell line. Next, to determine the underlying mechanism by which dog p21 is expressed as two isoforms, 5’ and 3’ rapid amplification of cDNA ends were performed to identify the splicing variants of dog p21. We found that only one dog p21 mRNA was detectable (data not shown), suggesting that expression of two dog p21 isoforms is not due to alternative splicing. These results let us speculate that other mechanisms, such as protein phosphorylation, may contribute to expression of two dog p21 isoforms. In this regard, λ protein phosphatase was used to treat MDCK cell lysates, followed by Western blot analysis. Interestingly, we showed that the level of the slow migrating dog p21 was diminished upon treatment with λ protein phosphatase regardless of DNA-damaging agent treatment, suggesting that the upper band is a phosphorylated isoform of dog p21 (Fig. 1B, compare lanes 1, 3, and 5 with 2, 4, and 6, respectively). Together, these
data suggest that phosphorylation is responsible for expression of two p21 isoforms in dog.

Serine 123 Phosphorylation Is Required for Expression of the Phosphorylated p21 Isoform—To identify the region in dog p21 responsible for the two-isoform expression, we constructed several chimeric proteins, where individual regions of dog and human p21 were replaced with their corresponding regions from human and dog p21, respectively (Fig. 2, A and B). Upon transfection into Cf2Th cells, all of the chimeric proteins were efficiently expressed (Fig. 2C). Interestingly, we found that dog...
p21(Hu 117–126) chimeric protein, where the region from amino acid 117 to 126 in dog p21 was substituted with that from human p21, was expressed as one isoform, which migrated close to the underphosphorylated dog p21 isoform (Fig. 2C, lane 6). To verify that the region from amino acid 117 to 126 in dog p21 is responsible for expression of the phosphorylated dog p21 isoform, we generated two more human p21 chimeric proteins, human p21(Dog 117–136) and human p21(Dog 117–126), where the region from amino acid 117 to 126 or 136 in human p21 was replaced with its corresponding region from dog p21. Upon expression, we found that unlike the wild-type human p21, both human p21(Dog 117–136) and human p21(Dog 117–126) were expressed as two isoforms (Fig. 2D, compare lane 2 with lanes 3–4). In addition, the upper bands of human p21(Dog 117–136) and human p21(Dog 117–126) were diminished upon λ phosphatase treatment (Fig. 2E, compare lanes 1 and 3 with 2 and 4, respectively). These data suggest that the region from amino acid 117 to 126 in dog p21 is required for expression of the phosphorylated dog p21 isoform.

Notably, in the region from amino acid 117 to 126, four amino acids are different between human and dog p21, which are leucine at 120, histidine at 122, proline at 124, and arginine at 126 (Fig. 2A). Thus, these four amino acids in dog p21 were individually replaced with their corresponding residues from human p21, and the resulting mutants were named as dog p21(L120V), dog p21(H122R), dog p21(P124G), and dog p21(R126Q). We found that dog p21(P124G) variant was expressed as one isoform, migrating close to the underphosphorylated dog p21 (Fig. 2F, lane 5). By contrast, the other three variants, L120V, H122R, and R126Q, were still expressed as two isoforms (Fig. 2F, lanes 3–4 and 6). To verify that proline 124 is required for expression of the phosphorylated dog p21 isoform, we generated a human p21(G124P) variant, where the glycine at 124 in human p21 was substituted with a proline. We found that the human p21(G124P) variant was expressed as two isoforms (Fig. 2D, lane 6), of which the high molecular mass band was sensitive to λ phosphatase treatment (Fig. 2E, compare lanes 7 and 8). As a control, we showed that human p21(R122H) variant, where arginine 122 was replaced with a histidine, was expressed as one isoform although it migrated slower than wild-type human p21 (Fig. 2D, compare lane 2 with 5).

It is known that although proline itself cannot be phosphorylated, it can mediate phosphorylation of a preceding serine or threonine by a proline-directed kinase. Indeed, dog p21 contains a serine in front of proline 124. Thus, serine 123 is likely to be the phospho-acceptor amino acid. To test this, the serine 123 in dog p21 was substituted with a nonphosphorylatable alanine (S123A) or a phosphomimetic amino acids aspartate (S123D). Upon expression, we found that both dog p21(S123A) and dog p21(S123D) was expressed as one isoform (Fig. 2G, lanes 3–4). Interestingly, dog p21(S123A) migrated around 19 kDa, similar to that of dog p21(Hu 117–126) or the underphosphorylated isoform of dog p21 (Fig. 2G, compare lane 3 with lanes 1–2). By contrast, dog p21(S123D) migrated close to the phosphorylated isoform of dog p21 (Fig. 2F, compare lane 1 with 4). Taken together, these data suggest that serine 123 phosphorylation is responsible for expression of the phosphorylated dog p21 isoform.
upper band, was approximately 50 min (Fig. 4C). Taken together, these data suggest that serine 123 phosphorylation increases the protein stability of dog p21.

**Serine 123 Phosphorylation Prevents Dog p21 from Ubiquitin-independent Proteasomal Degradation**—To investigate further how serine 123 phosphorylation enhances dog p21 protein stability, we examined whether phosphorylation of serine 123 inhibits dog p21 protein degradation. In this regard, the proteasome inhibitor MG132 was used. We showed that the level of ectopic wild-type, S123A, and S123D dog p21 was elevated upon treatment with MG132 (Fig. 5A, compare lanes 1, 2, and 3 with 4, 5, and 6, respectively). Consistent with this, both endogenous dog p21 isoforms were also stabilized by MG132 (Fig. 5B, compare lane 1 with 2). These data suggest that dog p21, like its human counterpart, is degraded by a proteasomal pathway.

Next, we sought to determine whether ubiquitylation plays a role in the proteasomal degradation of dog p21. To address this, the ts20 cell line was used. ts20 cells contain a temperature-sensitive E1 ubiquitin-activating enzyme (36), and thus the ubiquitin-dependent proteasomal pathway is inactivated at the nonpermissive temperature (39 °C). Specifically, ts20 cells were transfected with wild-type, S123A, and S123D dog p21 and then cultured at 35 °C (permissive temperature) or 39 °C (nonpermissive temperature) to inactivate E1, followed by cycloheximide treatment for various times. We found that at 35 °C, wild-type, S123A, and S123D dog p21 were able to be degraded through the ubiquitin-dependent pathway (Fig. 5, C–E, left p21 panels). As a positive control, p53 protein was found to be degraded through the ubiquitin-dependent pathway (Fig. 5, C–E, left p53 panels). Importantly, we found that upon inacti-
vation of E1, the phosphorylated dog p21 isoform, the upper band, was stably expressed, whereas the unphosphorylated dog p21, the lower band, was degraded rapidly (Fig. 5 C, right p21 panel). Consistent with this, dog p21(S123D), mimicking the phosphorylated dog p21 isoform, was stable (Fig. 5 D, right p21 panel), whereas dog p21(S123A), mimicking the underphosphorylated dog p21 isoform, was degraded rapidly (Fig. 5 E, right p21 panel). Together, these data suggest that serine 123 phosphorylation prevents dog p21 from ubiquitin-independent proteasomal degradation.

**DISCUSSION**

We have previously shown that dog p21 is expressed as two isoforms, one high molecular mass band of 23 kDa and one low molecular mass band of 19 kDa (32). In the current study, we found that expression of two dog p21 isoforms is due to proline-directed phosphorylation at serine 123 (Figs. 1 and 2). Notably, in dog p21, serine 123 is followed by a proline at codon 124. Thus, these two residues form a consensus site for the proline-directed phosphorylation. Indeed, substitution of either serine 123 with alanine or proline 124 with glycine impairs the phosphorylation site, leading to expression of one dog p21 isoform (Fig. 2, E and F). We would like to mention that only serine 123, but not proline 124, is conserved between human and dog p21 as human p21 contains a glycine at position 124. Thus, when the glycine 124 was replaced with a proline in human p21, the resulting human p21 variant, called p21(G124P), can be phosphorylated and is expressed as two isoforms like dog p21 (Fig. 2, C and D). Together, our data suggest that proline-directed serine 123 phosphorylation leads to expression of two dog p21 isoforms. To our knowledge, this is the first phosphorylation site identified so far results in a significant upshift of p21 protein.

In our study, we showed that dog p21(S123A), mimicking the underphosphorylated isoform, is less efficient in blocking S phase entry compared with that by wild-type dog p21 or dog p21(S123D), mimicking the phosphorylated isoform (Fig. 3 B). Consistent with this, we also showed that dog p21(S123A)-mediated growth suppression is less robust than that by wild-type or mutant S123D (Fig. 3, C–F). These data suggest that phosphorylation of serine 123 is critical for dog p21 to inhibit cell proliferation, at least in part by preventing S phase entry. Human p21 exerts its ability to block S phase entry mainly by inhibiting the activity of the cyclin-CDK2 complex, resulting in less Rb phosphorylation (6, 7, 37). Similarly, dog p21(S123D) is more potent than dog p21(S123A) in suppressing Rb phosphorylation (Fig. 3 A). Thus, future studies are needed to elucidate how serine 123 phosphorylation enhances the ability of dog p21 in growth suppression. Furthermore, phosphorylation of human p21 was reported to promote G2-prophase transition (38). However, both underphosphorylated and phosphorylated dog p21 were increased to a similar density upon aphidicolin blockade release (data not shown), consistent with previous report (39). These results suggest that serine 123 phosphorylation is not regulated in a cell cycle-dependent manner.

Like human p21, we found that dog p21 is a short life protein (Fig. 4). Interestingly, we found that the half-life of phosphorylated dog p21 is much longer than that of the underphosphorylated isoform. These results indicate that serine 123 phosphor-

**FIGURE 4. Phosphorylation of serine 123 increases the stability of dog p21 protein.** A, CEFTh cell expressing wild-type dog p21, dog p21(S123A), or dog p21(S123D) were mock-treated or treated with cycloheximide for various times, followed by Western blot analysis to determine the level of p21 proteins and actin. B and C, left panels, MDCK cells were mock-treated or treated with cycloheximide for various times followed by Western blot analysis to determine the level of p21 proteins and actin. Right panels, upon normalization to actin, the relative half-life of underphosphorylated dog p21 (B) and phosphorylated dog p21 (C) was calculated.
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Phosphorylation of serine 123 in dog p21 and to determine whether serine 123 phosphorylation affects the cellular localization of dog p21.

In our study, we found that fast migrating dog p21 and dog p21(S123A), but not the slow migrating dog p21 and dog p21(S123D), can be degraded via the ubiquitin-independent pathway (Fig. 5, C–E). This suggests that phosphorylation of serine 123 prevents dog p21 from ubiquitin-independent proteasomal degradation, which may be responsible for the increased protein stability of dog p21. Human p21 is known to be degraded via both ubiquitin-dependent and -independent pathways. Specifically, several E3 ligases, including SCFSKp2, CRL4CDT2, and APC/C, are found to target human p21 for ubiquitin-dependent degradation (24, 25, 27). In addition, p21 can be degraded through the ubiquitin-independent pathway via binding with Mdm2 E3 ligase or interaction with the C8 proteasome subunit (26, 41, 42). Thus, further studies are warranted to address how serine 123 phosphorylation affects ubiquitin-dependent and -independent pathways.

**Acknowledgment**—We thank Yuqian Jiang for technical support.

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**FIGURE 5. Serine 123 phosphorylation prevents dog p21 degradation through ubiquitin-independent pathway.** A, Cf2Th cells expressing wild-type dog p21, dog p21(S123A), and dog p21(S123D) were mock-treated or treated with MG132 for 12 h followed by Western blot analysis to determine the level of p21 and actin. B, MDCK cells were mock-treated or treated with MG132 for 12 h followed by Western blot analysis to determine the level of dog p21 proteins, p53, and GAPDH.
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