ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 120–125

BBRC

www.academicpress.com

Identification of the regulatory region required for ubiquitination of the cyclin kinase inhibitor, p21

Kunihiko Fukuchi,[a,b,*] Tamio Hagiwara,[a] Kentarou Nakamura,[a] Sachiko Ichimura,[c] Kouichi Tatsumi,[c] and Kunihide Gomi[b]

[a] *Department of Biochemistry, Showa University, School of Medicine, Tokyo 142-8555, Japan*
[b] *Department of Clinical Pathology, Showa University, School of Medicine, Tokyo 142-8555, Japan*
[c] *Research Center for Radiation Safety, National Institute of Radiological Sciences, Chiba 263-8555, Japan*

Received 12 March 2002

Abstract

The expression of cyclin kinase inhibitor p21 is regulated by the ubiquitin-proteasome protein degradation system, as well as by transcriptional regulation. Generally, ubiquitination is regulated by the phosphorylation of the substrate. In this study, we identified the region of p21 responsible for the regulation of ubiquitination. Since the phosphorylation sites of p21 are distributed in the C-terminal region, we constructed sequential C-terminal truncated fragments and examined their ubiquitination in eukaryotic cells. The ubiquitination was observed in the 1–164 (full length) and 1–157 fragments with the same efficiency, but not in the 1–147 fragment. The lack of ubiquitination in the 1–147 fragment was unlikely due to the removal of a Lys residue at position 154, since the p21 K154R mutant was ubiquitinated as efficiently as the full-length p21. Furthermore, the 148–157 deleted form of p21 was not ubiquitinated, just like the 1–147 fragment. Thus, the C-terminal 148–157 region, not a ubiquitination site by itself, should contain an essential regulatory region for the efficient ubiquitination of p21.

Keywords: Cyclin kinase inhibitor; p21; Ubiquitin; PCNA; Truncated fragment

The progression of the cell cycle is precisely controlled by cyclin dependent kinases (CDKs). The sequential activation of CDKs is accomplished by the association of each CDK with a specific cyclin subunit [1,2]. In the mammalian cell cycle, G1 progression is driven by the association of cyclinD with either CDK4 or CDK6, while the G1–S transition requires both cyclinE–CDK2 and cyclinA–CDK2 associations [1]. As the cell cycle progresses, each cyclin is degraded, and the phase specific CDK activity is diminished [3,4]. However, to ensure the prevention of an unscheduled entry into S phase, the activity of the cyclin–CDK complex is suppressed by an association with CDK inhibitors [5]. For control at the G1–S checkpoint, there are two groups of CDK inhibitors, the p21 family and the p16INK4 family. Ample evidence suggests that the expression of cyclin kinase inhibitors is regulated at both the transcriptional and protein levels [5,6].

When dividing cells are exposed to genetic stress, the cell cycle must be arrested immediately to ensure the integrity of the DNA and/or cell cycle control. Cyclin kinase inhibitors are constitutively expressed in cells to prepare for their prompt response against genetic stress during G1. If the cell cycle progresses from G1 to S on schedule and without DNA damage, the inhibitors are degraded through the ubiquitin-proteasome pathway, as are cyclins [7]. Consequently, DNA damage may result in the accumulation of cyclin kinase inhibitors through the inhibition of their degradation.

The p21, a transcriptional target of p53, inhibits cell cycle progression through its interactions with cyclin–CDK complexes and proliferating cell nuclear antigen (PCNA) [8,9]. In addition, p21 is induced by p53 independent signals during growth arrest and cellular differentiation [10]. Zeng and el-Deiry [11] demonstrated that p21 expression is regulated by both transcriptional and posttranscriptional mechanisms. As for posttranscriptional mechanisms, caspase digestion and the ubiquitin-proteasome protein degradation system have

* Corresponding author. Fax: 81-3-3784-2346.
E-mail address: kfukuchi@med.showa-u.ac.jp (K. Fukuchi).

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00198-5

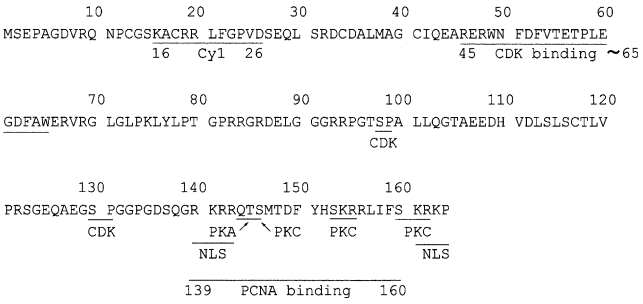

Fig. 1. The phosphorylation sites and binding motifs of p21.

been suggested. In caspase digestion, cleavage at the C-terminal Asp-112 by caspase3 during DNA damage induced apoptosis was reported [12–14]. Involvement of the ubiquitin-proteasome pathway has been demonstrated by the stabilization of p21 by proteasome inhibitors and by the presence of ubiquitinated p21 [15–18]. In support of the participation of the ubiquitin proteasome, the SCF complex for p21 was observed in p21 degradation [19]. In contrast, Sheaff et al. [20], using a nonubiquitinated form of p21, demonstrated that the direct ubiquitination of p21 is not required for its turnover by the proteasome.

Nevertheless, since the ubiquitination and degradation of p21 by the proteasome system are evident, the regulatory mechanism of ubiquitination needs to be elucidated. Generally, ubiquitination is controlled by the phosphorylation of a substrate protein [21]. Phosphorylation sites of p21 have been reported at Ser-98 and Ser-130 by CDK [22], Thr-145 and Ser-146 by cAMP-dependent protein kinase (PKA), and Ser-153 and Ser-160 by protein kinase C (PKC) as indicated in Fig. 1 [23].

In this study, we investigated the critical regulatory region required for the ubiquitination of p21. To accomplish this, C-terminal or N-terminal truncated fragments of p21 were expressed in eukaryotic cells, and their ubiquitination was examined.

Materials and methods

Generation of expression plasmids. The DNA fragments coding truncated or deleted p21 fragments were propagated by PCR using a p21 cDNA clone as a template [24]. The generated fragments were inserted into either pcDNA3.1/His or pcDNA3.1/Myc-His vectors (Invitrogen, CA), and transfected into JM109 according to manufacturer's instruction. The transfectants were propagated and extracted as described previously [25]. A portion of the plasmid DNA (3 μg) was digested with six units of *Sal*I restriction endonuclease for use in DNA sequencing. Sequencing reactions were performed using the dideoxynucleotide termination method and the M13 universal primer 5′CGACGTTGTAAAACGACGGCCAGT3′. Results were analyzed by an ALF sequencer (Pharmacia, Sweden) as described previously [26]. The sequence obtained was compared with the p21 cDNA sequence to confirm that the correct clone is constructed. The p21 K154R mutant was generated using a site-directed mutagenesis kit (Clontech, CA).

Expression of truncated p21. The human cervix carcinoma cell line, HeLa, and the human colon cancer cell line, DLD-1, from the Health Science Research Resource Bank were used for expression of the truncated p21 fragments. The cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 incubator. Cells were seeded at 1×10^6 cells in 10 cm diameter dishes, and passaged every third day. Exponentially growing cells (2×10^7) were harvested, washed with ice-cold phosphate buffered saline (PBS), and then resuspended in 0.5 ml of DMEM without FCS on ice. Cell suspensions were transferred to electroporation cuvettes (Bio Rad, CA) that were chilled on ice. Supercoiled plasmid DNA (180 μg) was added to the cell suspension, and the mixture was incubated on ice for 5 min. Electroporation was performed at 250 V with a 1000 μF capacitor. After electroporation, the cuvette was placed on the ice for 10 min. Transfected cells were diluted into 10 ml DMEM with 10% FCS, and cultured at 37 °C in a humidified 5% CO_2 incubator for 48 h.

Protein extraction and Western blot analysis. A total of 2×10^7 cells was harvested by trypsinization, washed with PBS, and then lysed in 4 ml of 6 M guanidium hydrochloride, 2×10^{-2} M sodium phosphate, 5×10^{-1} M NaCl, pH 7.8. The lysate was homogenized by passing through a 21-gauge needle four times. Approximately 200 μl of a 50% slurry ProBond resin (Invitrogen) was added to the lysate, and the mixture was rocked gently for 1 h. The resin was subjected to the following series of washes: twice with 8 M urea, 2×10^{-2} M sodium phosphate, and 5×10^{-1} M sodium chloride, pH 7.8; twice with 8 M urea, 2×10^{-2} M sodium phosphate, and 5×10^{-1} M sodium chloride, pH 6.0; and twice with 8 M urea, 2×10^{-2} M sodium phosphate, and 5×10^{-1} M sodium chloride, pH 5.3. After washing the resin, the recombinant protein was eluted using 8 M urea, 2×10^{-2} M sodium phosphate, and 5×10^{-1} M sodium chloride, pH 4.0. The eluents were dialyzed against 5×10^{-3} M Tris, pH 8.0, and concentrated by vacuum concentration. For Western blot analysis, 1% sodium dodecyl sulfate (SDS), and 2.5% β-mercaptoethanol were added to the eluent, and the mixture was heated for 5 min at 100 °C. The denatured samples were subjected to 10–20% SDS–polyacrylamide gel electrophoresis using a running buffer of 2.5×10^{-2} M Tris–HCl, pH 8.3, 1.92×10^{-1} M glycine, 0.1% SDS. The gels were electroblotted (5 mA/cm^2, 2 h) onto a polyvinylidene difluoride (PVDF) membrane (Bio Rad) in a transfer buffer of 2.5×10^{-2} M Tris–HCl, pH 8.3, 1.92×10^{-1} M glycine, and 20% methanol. The membranes were incubated with 10% nonfat dry milk in 1×10^{-2} M Tris–HCl, pH 8.3, 5×10^{-1} M NaCl (TBS) for 1 h at 25 °C, and then incubated with either anti-p21 rabbit polyclonal antibody (1:200; H164, Santa Cruz Biotechnology, CA), anti-Myc monoclonal antibody (1:1000; R950-25, Invitrogen), anti-Xpress monoclonal antibody (1:1000; R910-25, Invitrogen), or anti-ubiquitin monoclonal antibody (1:1000; FK2, Medical & Biological Laboratories, Japan) diluted in TBS for 1 h at 25 °C. The blot was washed in TBS with 0.05% Tween20 (TTBS) to remove the unbound antibody and incubated with a biotinylated anti-rabbit IgG or biotinylated anti-mouse IgG in TBS for 1 h at room temperature. The membrane was again washed in TTBS and incubated in streptavidine–biotinylated alkaline phosphatase complex. After washing thoroughly, nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate was used to visualize the bands.

Results

Construction of the C-terminal truncated fragments

In many of the ubiquitin-proteasome protein degradation pathways, the recognition of the substrate protein by E3 ubiquitin ligase is regulated by the phosphorylation of the substrate protein [21,27]. In the

case of p21, several serine or threonine residues have been reported to be sites of phosphorylation. These sites include the serine–proline motifs at Ser-98 and Ser-130 that are phosphorylated by CDKs [22], the Ser-146, Ser-153, and Ser-160 that are phosphorylated by PKC, and Thr-145 that is phosphorylated by PKA (Fig. 1) [23]. Therefore, we constructed C-terminal truncated fragments with C-terminal Myc tags, named pMyc128, 147, 157, or 164, that delete each phosphorylation site in the mammalian expression plasmid, pcDNA3.1/Myc-His (Fig. 2A). In addition, we constructed C-terminal truncated fragments with N-terminal Xpress tags, named pXpress128, 147, 157, or 164, that delete each phosphorylation site in the mammalian expression plasmid, pcDNA3.1/His (Fig. 3A).

Ubiquitination of C-terminal truncated p21 fragments

The expression plasmids, pMyc128, pMyc147, pMyc157, and pMyc164, were transfected into DLD-1 or HeLa cells by electroporation. Recombinant proteins were purified by immobilized metal affinity chromatography using ProBond resin. To confirm that the purified protein was the product of the transfected plasmid,

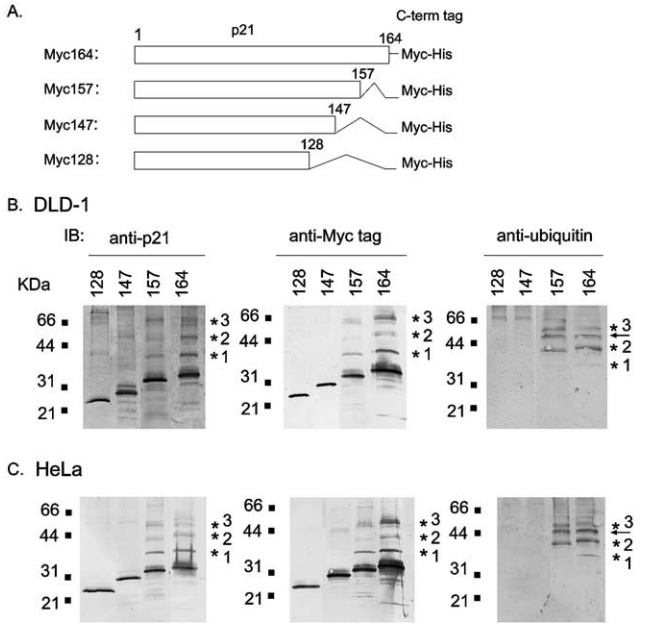

Fig. 2. The ubiquitination of C-terminal tagged p21 C-terminal truncated fragments. The constructions of Myc tagged C-terminal truncated fragments are indicated in panel A. The DNA fragments encoding the truncated fragments were cloned into pcDNA3.1/Myc-His. For transient expression, 180 μg of supercoiled DNA was electroporated into exponentially growing 2×10^7 DLD-1 or HeLa cells. After 48 h cultivation, the cells were lysed in guanidine, and the recombinant proteins were purified by metal affinity choromatography using ProBond. Concentrated purified proteins were applied to Western blot analysis using anti-p21, anti-Myc, or anti-ubiquitin antibodies. Expressions in DLD-1 cells are shown in panel B and HeLa cells in panel C.

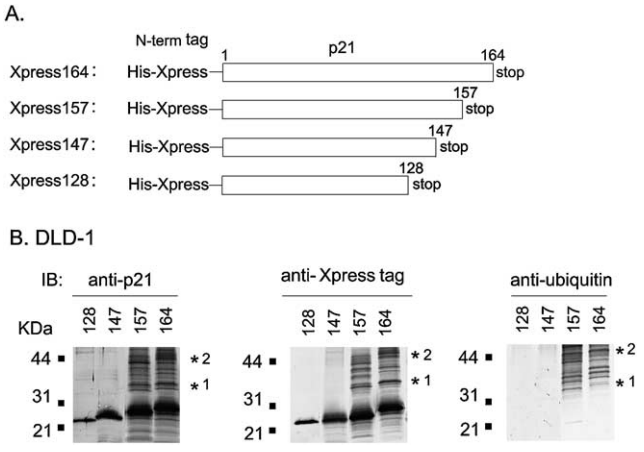

Fig. 3. The ubiquitination of N-terminal tagged p21 C-terminal truncated fragments. The constructions of Xpress tagged C-terminal truncated fragments are indicated in panel A. The DNA fragments encoding the truncated fragments were cloned into pcDNA3.1/His. For transient expression, 180 μg of supercoiled DNA was electroporated into exponentially growing 2×10^7 DLD-1 cells. After 48 h cultivation, the cells were lysed in guanidine and the recombinant proteins were purified by metal affinity choromatography using ProBond. In panel B, concentrated purified proteins were subjected to Western blot analysis using anti-p21, anti-Myc, or anti-ubiquitin antibodies.

Western blot analysis of the recombinant proteins using anti-p21 and anti-Myc antibodies was performed. All the expression plasmids yielded protein products with the expected molecular weights (Figs. 2B and C left and center panels). In the purified protein of pMyc157 and pMyc164 transfected cells, extra bands that reacted with anti-p21 and anti-Myc tag were observed at 31–66 kDa. To confirm that the extra bands represent ubiquitinated p21 fragments, the same samples were blotted and probed with anti-ubiquitin antibody FK2. The higher molecular weight bands, indicated as *2 and *3, were detected by anti-p21, anti-Myc, and anti-ubiquitin antibodies. Since the anti-ubiquitin monoclonal antibody, FK2, specifically recognizes the multi-ubiquitin chain [28], the band detected with the anti-p21 antibody or the anti-Myc tag antibody, indicated as "*1" in Figs. 2B and C left and center panels that was considered a tagged p21 fragment with a single ubiquitin molecule, was detected as either a faint band or not detected at all with the FK2 antibody. The patterns of Western blots obtained in DLD-1 and HeLa cells were identical therefore these observations did not depend on the cell type.

The structural modification by the presence of the Myc tag at the C-terminal may affect the ubiquitination of recombinant proteins. Therefore, we performed the same analysis using N-terminal Xpress tag-truncated p21 fragments (Fig. 3A). The results obtained using DLD-1 cells are shown in Fig. 3. Xpress164 and Xpress157 showed higher molecular bands on the blot probed by either anti-p21 antibody or anti-Xpress antibody, indicated as *1 and *2 (Fig. 3B left and center). Using the anti-ubiquitin antibody, bands *1 and *2 were

detected. The same patterns were obtained when using HeLa cells (data not shown). The result that the ubiquitinated bands detected for pMyc157 and pMyc164 but not for pMyc147 and pMyc128 indicates that the critical element for ubiquitination locates in the 148–157 region.

The region (148–157) is indispensable for p21-ubiquitination

Since the 148–157 aa fragment includes a lysine residue at 154, the loss of ubiquitination of the 1–147 fragment may be due to the removal of the lysine residue as the ubiquitination site. To identify whether Lys-154 is the ubiquitination site, we constructed the Lys-154 to Arg mutant, pMycK154R, using pMyc164 as a template and examined its ubiquitination. pMyc164 or pMycK154R was transfected into DLD-1 cells and the metal affinity purified cell lysate was probed with anti-p21 antibody. The higher molecular weight bands were detected in the MycK154R lysate at levels similar to the wild type full-length p21 in the Myc164 lysate (Fig. 4A), indicating that Lys-154 is not the ubiquitination site. Next, to confirm the significance of 148–157 region, we constructed the 148–157 deleted form of p21, MycΔ148–157. After transfection of pMyc147, pMyc157, or pMycΔ148–157 into DLD-1 cells, the presence of ubiquitinated products was examined. As indicated in Fig. 4B, higher molecular weight bands were observed only in Myc164, but not in Myc147 and MycΔ148–157. Thus, 148–157 region is an indispensable regulatory region for ubiquitination.

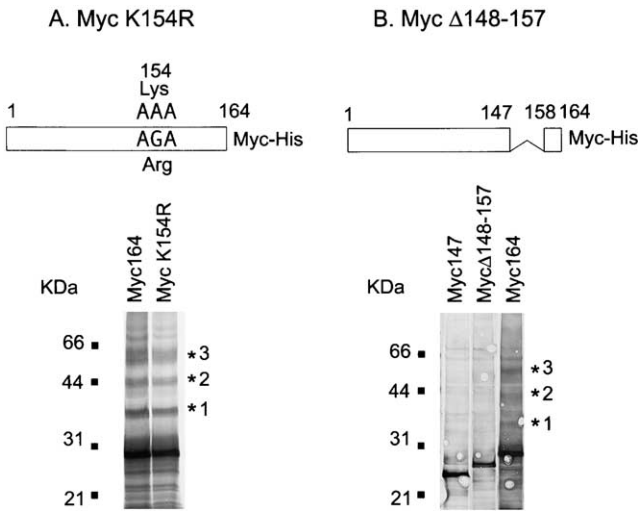

Fig. 4. The ubiquitination of the p21 K154R mutant or 148–157 deleted form of p21. (A) The construction of Lys-154 to Arg mutant of Myc164, MycK154R is indicated at the top. (B) The construction of the 148–157 deleted form of p21, MycΔ148–157 is indicated at the top. Each construct was transfected into exponentially growing 2×10^7 DLD-1 cells. After 48 h cultivation, the cells were lysed in guanidine and the recombinant proteins were purified by metal affinity choromatography using ProBond. Concentrated purified proteins were subjected to Western blot analysis using anti-p21 antibody.

Ubiquitination of N-terminal truncated p21 fragments

Lysine residues exist in p21 at 16, 75, 141, 154, 161, and 163. To identify the lysine or the N-terminal structure required for ubiquitination, we constructed two N-terminal deletion fragments, a 20 aa-deletion fragment (Xpress21C) and an 84 aa-deletion fragment (Xpress85C) (Fig. 5A). On Western blots obtained after metal affinity purification, anti-p21 and anti-Xpress tag antibodies detected tagged p21 fragments and higher molecular bands indicated as *1, *2, and *3 (Fig. 5B). These higher molecular bands, *2 and *3, were detected using the anti-ubiquitin antibody FK2 (Fig. 5B). When Xpress85C was expressed in cells, only the tagged truncated fragments were detected by anti-p21 or anti-Xpress antibodies (Fig. 5C).

Discussion

Using the expression of C-terminal truncated p21 fragments, p21 K154R mutant, and the 148–157 deleted form of p21 in vivo, we have demonstrated that the C-terminal 148–157 aa region is required for the efficient ubiquitination of p21.

The significance of the C-terminal region for the ubiquitination of p21 has been demonstrated previously. Cayrol and Ducommun [29] reported that the binding of PCNA to the C-terminal region of p21 stabilized the p21 protein by inhibiting its ubiquitination. The region

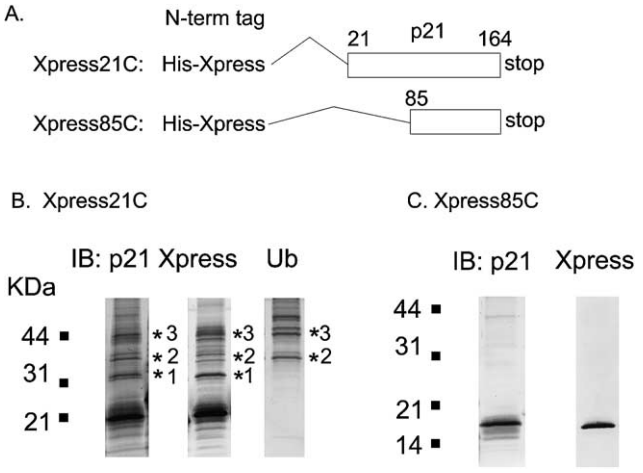

Fig. 5. The ubiquitination of N-terminal tagged p21 N-terminal truncated fragments. The constructions of Xpress tagged N-terminal truncated fragments are indicated in panel A. The DNA fragments encoding the truncated fragments were cloned into pcDNA3.1/His. For transient expression, 180 μg of supercoiled DNA was electroporated into exponentially growing 2×10^7 DLD-1 cells. After 48 h cultivation, the cells were lysed in guanidine, and the recombinant proteins were purified by metal affinity choromatography using ProBond. In panel B, concentrated purified proteins were subjected to Western blot analysis using anti-p21, anti-Myc, or anti-ubiquitin antibodies.

important in the binding of PCNA to p21 has been localized to 22 residues of the 139–160 aa peptide of the C-terminus of p21 by crystallography [30].

There are several sites in the C-terminal region of p21 that are phosphorylated by either PKC or PKA (Fig. 1). Most of the ubiquitination signal is attributed to the phosphorylation of the target protein [21]. We demonstrated that the 1–157 fragment was as efficiently ubiquitinated as full-length p21, but that the 1–147 fragment was not ubiquitinated, and that Lys-154 in the 148–157 fragment was not the ubiquitinated lysine residue. These results suggest that the region between 148 and 157 aa is an essential regulatory region for the ubiquitination of p21.

Cayrol and Ducommun [29] reported that a p21 mutant deficient for the interaction of p21 with CDKs displayed an enhanced stability and a greatly reduced sensitivity to proteasome-mediated proteolysis, suggesting that an association with cyclin–CDK complexes may trigger p21 degradation. According to these data, phosphorylation by CDK is expected to be a ubiquitination signal. However, our present data indicate that the CDK phosphorylation sites at Ser-98 or Ser-130 are not essential regulatory sites for the ubiquitination of p21.

Lysine residues have been identified at amino acid 16, 75, 141, 154, 161, and 163. In the present study, ubiquitination was observed in a 20 amino acid residue deletion at the N-terminus, but was not observed in an 84 amino acid deletion at N-terminus. In turn, efficient ubiquitination was observed in the 1–157 aa fragment. These results suggest that the N-terminal structure formed by 1–84 aa is essential for efficient ubiquitination.

In the present data, the 1–147 aa fragment was not ubiquitinated, however, the ubiquitination of 1–82 aa was reported previously [31]. The difference between their conditions and ours is the origin of ubiquitin. In our experiments, we used the endogenous ubiquitin, while they used a forced expressed His-tagged ubiquitin. In our experimental conditions, the level of ubiquitination may be lower than that reported by Rousseau et al. [31].

The inhibiting effects on the cell cycle by DNA damage have been demonstrated as an arrest at the G1–S checkpoint and as a retardation of S phase progression. Ogryzko et al. [32] reported that p21 retards S phase progression through the inhibition of cyclin A or E. It was evident that p21 forms quaternary complexes with cyclinA, CDK2, and PCNA [22,33]. Further, phosphorylation of C-terminal regulatory region of p21 inhibits the PCNA–p21 binding [23]. Our result, together with others, suggests that the association of DNA damage and p21 is as follows. DNA damage inactivates a molecular species of PKC or dephosphorylates p21. PCNA binds to the dephosphorylated p21 to stabilize it by inhibiting ubiquitination. The retarded S phase caused by the stabilization of p21 may provide the occasion to repair damaged DNAs.

Acknowledgments

Part of this work was supported by a Grant-in Aid from the Ministry of Education, Culture, Sports, Science and Technology and the Japan Private School Foundation.

References

[1] C.J. Sherr, G1 phase progression: cycling on cue, Cell 79 (1994) 551–555.
[2] D.O. Morgan, Cyclin-dependent kinases: engines, clocks, and microprocessors, Annu. Rev. Cell Dev. Biol. 13 (1997) 261–291.
[3] T. Hunter, J. Pines, Cyclins and Cancer II: cyclin D and CDK inhibitors come of age, Cell 79 (1994) 573–582.
[4] M. Glotzer, A.W. Murray, M.W. Kirschner, Cyclin is degraded by the ubiquitin pathway, Nature 349 (1991) 132–138.
[5] C.J. Sherr, J.M. Roberts, Inhibitors of mammalian G1 cyclin-dependent kinases, Genes Dev. 9 (1995) 1149–1163.
[6] M. Pagano, S.W. Tam, A.M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P.R. Yew, G.F. Draetta, M. Rolfe, Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27, Science 269 (1995) 682–685.
[7] D.M. Koepp, J.W. Harper, S.J. Elledge, How the cyclin became a cyclin: regulated proteolysis in the cell cycle, Cell 97 (1999) 431–434.
[8] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarsi, S.J. Elledge, The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, Cell 75 (1993) 805–816.
[9] W.S. el-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, B. Vogelstein, WAF1, a potential mediator of p53 tumor suppression, Cell 75 (1993) 817–825.
[10] D.A. Liebermann, B. Hoffman, R.A. Steinman, Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways, Oncogene 11 (1995) 199–210.
[11] Y.X. Zeng, W.S. el-Deiry, Regulation of p21WAF1/CIP1 expression by p53-independent pathways, Oncogene 12 (1996) 1557–1564.
[12] F. Chai, A. Evdokiou, G.P. Young, P.D. Zalewski, Involvement of p21 (Waf1/Cip1) and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate, Carcinogenesis 21 (2000) 7–14.
[13] J.L. Gervais, P. Seth, H. Zhang, Cleavage of CDK inhibitor p21 (Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis, J. Biol. Chem. 273 (1998) 19207–19212.
[14] B. Levkau, H. Koyama, E.W. Raines, B.E. Clurman, B. Herren, K. Orth, J.M. Roberts, R. Ross, Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade, Mol. Cell 1 (1998) 553–563.
[15] C.G. Maki, J.M. Huibregtse, P.M. Howley, In vivo ubiquitination and proteasome-mediated degradation of p53(1), Cancer Res. 56 (1996) 2649–2654.
[16] M.V. Blagosklonny, G.S. Wu, S. Omura, W.S. el-Deiry, Proteasome-dependent regulation of p21WAF1/CIP1 expression, Biochem. Biophys. Res. Commun. 227 (1996) 564–569.
[17] C.G. Maki, P.M. Howley, Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation, Mol. Cell. Biol. 17 (1997) 355–363.

[18] K. Fukuchi, S. Tomoyasu, T. Nakamaki, N. Tsuruoka, K. Gomi, DNA damage induces p21 protein expression by inhibiting ubiquitination in ML-1 cells, Biochim. Biophys. Acta 1404 (1998) 405–411.
[19] Z.K. Yu, J.L. Gervais, H. Zhang, Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins, Proc. Natl. Acad. Sci. USA 95 (1998) 11324–11329.
[20] R.J. Sheaff, J.D. Singer, J. Swanger, M. Smitherman, J.M. Roberts, B.E. Clurman, Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination, Mol. Cell 5 (2000) 403–410.
[21] S.J. Elledge, J.W. Harper, The role of protein stability in the cell cycle and cancer, Biochim. Biophys. Acta 1377 (1998) M61–M70.
[22] H. Zhang, G.J. Hannon, D. Beach, p21-containing cyclin kinases exist in both active and inactive states, Genes Dev. 8 (1994) 1750–1758.
[23] M.T. Scott, N. Morrice, K.L. Ball, Reversible phosphorylation at the C-terminal regulatory domain of p21(Waf1/Cip1) modulates proliferating cell nuclear antigen binding, J. Biol. Chem. 275 (2000) 11529–11537.
[24] A. Noda, Y. Ning, S.F. Venable, O.M. Pereira-Smith, J.R. Smith, Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen, Exp. Cell Res. 211 (1994) 90–98.
[25] K. Fukuchi, M. Sudo, Y.S. Lee, A. Tanaka, M. Nonoyama, Structure of Marek's disease virus DNA: detailed restriction enzyme map, J. Virol. 51 (1984) 102–109.
[26] H. Watanabe, K. Fukuchi, Y. Takagi, S. Tomoyasu, N. Tsuruoka, K. Gomi, Molecular analysis of the Cip1/Waf1 (p21) gene in diverse types of human tumors, Biochim. Biophys. Acta 1263 (1995) 275–280.
[27] J.D. Laney, M. Hochstrasser, Substrate targeting in the ubiquitin system, Cell 97 (1999) 427–430.
[28] M. Fujimuro, H. Sawada, H. Yokosawa, Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins, FEBS Lett. 349 (1994) 173–180.
[29] C. Cayrol, B. Ducommun, Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21, Oncogene 17 (1998) 2437–2444.
[30] J.M. Gulbis, Z. Kelman, J. Hurwitz, M. O'Donnell, J. Kuriyan, Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA, Cell 87 (1996) 297–306.
[31] D. Rousseau, D. Cannella, J. Boulaire, P. Fitzgerald, A. Fotedar, R. Fotedar, Growth inhibition by CDK–cyclin and PCNA binding domains of p21 occurs by distinct mechanisms and is regulated by ubiquitin-proteasome pathway, Oncogene 18 (1999) 4313–4325.
[32] V.V. Ogryzko, P. Wong, B.H. Howard, WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases, Mol. Cell Biol. 17 (1997) 4877–4882.
[33] H. Zhang, Y. Xiong, D. Beach, Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes, Mol. Biol. Cell 4 (1993) 897–906.