Involvement of the Interaction between p21 and Proliferating Cell Nuclear Antigen for the Maintenance of G2/M Arrest after DNA Damage*

Received for publication, July 10, 2001, and in revised form, August 27, 2001
Published, JBC Papers in Press, September 14, 2001, DOI 10.1074/jbc.M106460200

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Although a major effect of p21, a cyclin-dependent kinase inhibitor, is considered to be exerted during G1 phase of the cell cycle, p21 gene knock-out studies suggested its involvement in G/M checkpoint as well. Here we demonstrate evidence that p21 is required for the cell cycle arrest at G2 upon DNA damage. We found that expression of wild-type p21 (p21WT), not mutant p21 (p21PCNA−) lacking the interaction with proliferating cell nuclear antigen (PCNA), caused G2 cell cycle arrest in p53-deficient DLD1 colon cancer cell line after the DNA damage by treatment with cis-diaminedichloroplatinum (II). We also found that p21WT was associated with Cdc2/cyclin B1 together with PCNA. Furthermore, coimmunoprecipitation experiments revealed that PCNA interacted with Cdc25C at the G2/M transition, and this interaction was abolished when p21WT was expressed presumably due to the competition between p21WT and Cdc25C in the binding to PCNA. These findings suggest that p21 plays a regulatory role in the maintenance of cell cycle arrest at G2 by blocking the interaction of Cdc25C with PCNA.

The cell cycle is regulated by two major checkpoints at G1-S and G2/M transition. The fidelity of genomic replication during DNA synthesis and cell division is ensured by checkpoint controls that prevent cell cycle progression when the DNA damage or incomplete DNA replication is detected (1). Thus, checkpoint loss would result in genomic instability and has been implicated in carcinogenesis (2). In fact, the p53 tumor suppressor gene, a major gatekeeper of cell cycle checkpoints, is mutated in a large fraction of human cancers (3). Cell cycle arrest in G1 caused by DNA damage or cellular senescence is mediated by p21, a cyclin-dependent kinase inhibitor, that is under the transcriptional control of p53 (4). Interestingly, although cells deficient in p21 proliferate normally, they are unable to maintain stable G1 arrest and initiate cell death program when exposed to DNA-damaging agents such as irradiation and anti-cancer drugs (5, 6). Circumstantial evidences indicate the involvement of p21 at the G2/M transition. For example, p21 mRNA in human fibroblasts show bimodal periodicity with peaks in G1 and G2/M (7) and that p21 protein reaccumulates in the nucleus at the onset of mitosis (8). In addition, inducible p21 expression caused cell cycle arrest at G1 and G2 (9) and p21 induced G2 arrest when it was induced at the beginning of S phase (10). These observations have suggested that p21 is also involved in G2/M checkpoint.

It is well established that p21 consists of at least two functional domains that bind to proliferating cell nuclear antigen (PCNA)1 and Cdk/cyclins (11–13). PCNA was initially identified as an auxiliary protein for DNA polymerase δ that is essential for DNA replication and repair (14–16). The role of PCNA in cell cycle regulation is suggested by the fact that polymerase δ is regulated by cell cycle proteins (17). In fact, PCNA was shown to interact with various Cdk-cyclin complexes (11, 16, 18). Thus, PCNA may act as a platform for multiple protein-protein interactions involved in replication, repair, recombination, and cell cycle regulation. Interestingly, the PCNA protein levels increased steadily through the entire cell cycle period and remained high at G2/M (19). Thus, PCNA may also be involved in G2 cell cycle control.

The G2 checkpoint has been extensively studied in the fission yeast and is known to involve a number of proteins including Cdc2/cyclin B, Cdc25, 14-2-3, Wee1, Chk1, Cds1/Rad53, and DNA damage sensor proteins (20, 21). Among these proteins, Cdc25, a dual phosphatase for Cdc2, plays a central role in the G2 checkpoint by controlling the phosphorylation status, thus its kinase activity, of Cdc2 (22–24). In human cells, there are three Cdc25 homologues, Cdc25A, -B, and -C (25–27). Whereas Cdc25A is involved in the G1 checkpoint, Cdc25B and Cdc25C are involved in the G2/M transition (28–34). Because the activity of Cdc25C is induced at the onset of mitosis and regulates Cdc2/cyclin B1, it is regarded as a major regulator of the G2 checkpoint, besides the fact that Cdc25C has the highest homology with the yeast Cdc25. Cdc2 is subject to multiple levels of regulation including periodic association with cyclin B, phosphorylation, dephosphorylation (35–38), and intracellular compartmentation (39–44). Cdc2 associates with cyclin B at the G2/M transition (38), and this complex is retained in an inactive state throughout S and G2 phases by phosphorylation of

* This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare, the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Japanese Health Sciences Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PCNA, proliferating cell nuclear antigen; PI, propidium iodide; Tet, tetracycline; Fen1, flap endonuclease 1; WT, wild type; CDDP, cis-diaminedichloroplatinum (II); HA, hemagglutinin; PBS, phosphate-buffered saline; Cdk, cyclin-dependent kinase.

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Cdc2 at Thr-14 and Tyr-15 by another kinase Wee1 (22, 36). Wee1 is activated by upstream kinases, Chk1 or Cds1 (45, 46), that are activated by damaged DNA (47–49) and unreplicated DNA (45, 50, 51), respectively. Although Cdc25 activates Cdc2/cyclin B by reversing the Wee1-mediated phosphorylation of Cdc2 (22, 23), this activity is suppressed by Chk1 and Cds1.

Fig. 1. Abolishment of cell cycle progression by p21 requires its interaction with PCNA. A, detection of p21 expression in DLD1 p21WT and DLD1 p21PCNA cells. Cell lysates were prepared from cultured cells in the presence (+ Tet) of tetracycline or in the absence of tetracycline for 96 h (− Tet) and probed with anti-HA antibody (detecting both wild type and PCNA− mutant form p21 proteins) by Western blotting. B, quantitation of cells expressing p21 proteins by flow cytometry. C, cell cycle analysis. Cells were stained with propidium iodide (PI) and subjected to the DNA content analysis using fluorescence-activated cell sorter. The percentage of cells accumulated at each cell cycle stage is indicated. Induction of p21 protein was done by culturing cells in the medium without tetracycline for 96 h. Note that expression of wild-type p21 (DLD1 p21WT, + Tet) arrested the cell cycle progression at both G1 and G2/M with a significant decrease in cells at S phase, whereas expression of mutant p21PCNA− (DLD1 p21PCNA−, − Tet) failed to do so.
through phosphorylation of Cdc25 at Ser-216 in the yeast (50, 52, 53). The phosphorylated Cdc25 is sequestered in the cytoplasm by the interaction with 14-2-3 (54–59). However, a small amount of Cdc25 was shown to reside in the nucleus at interphase, and moreover, most of Cdc25 stays in the nucleus throughout cell cycle in some cells (46, 47). Therefore, an additional mechanism is required to ensure the inactivation of Cdc2-cyclin B complex by preventing coincidental contacts with Cdc25.

In this study, we attempted to examine the role of p21 in G2/M transition particularly in G2 DNA damage checkpoint by utilizing the p53-deficient human colon cancer cell lines inducibly expressing the wild-type or the mutant p21PCNA/H11002 lacking the interaction with PCNA specifically. Expression of p21 in these cells is under the tight control of a tetracycline-regulated promoter and can be induced by eliminating tetracycline (Tet) from the culture medium ("Tet-OFF" system). These cell lines were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum (IBL, Maebashi, Japan), 4 mM l-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), and 2 μg/ml tetracycline in a 5% CO2 incubator. Subcloning of these cell lines was repeatedly done in selective medium containing 50 μg/ml hygromycin. Western blotting and flow cytometry was performed to ensure that ~90% of the cells expressed p21 when tetracycline was eliminated. There was no significant difference in the sensitivity to CDDP among parental DLD1, DLD1 p21WT, and DLD1PCNA/H11002 cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Most of the chemicals including hygromycin, tetracycline, and cis-diaminedichloroplatinum (II) (CDDP) were purchased from Sigma. Fluorescein isothiocyanate-conjugated anti-HA (F-7), anti-PCNA (PC-10), anti-cyclin B1 (GNS1), anti-Cdc2 p34 (clone 17), anti-Cdk7 (C-14), and anti-cyclin H (FL-323) mouse monoclonal antibodies and anti-Cdc25C rabbit polyclonal antibody (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-HA mouse monoclonal antibody (3F10) was purchased from Roche Molecular Biochemicals. Biotin-conjugated anti-PCNA mouse monoclonal antibody was purchased from PharMingen (San Jose, CA). Secondary antibodies and horseradish peroxidase-conjugated goat anti-rabbit and sheep anti-mouse antibodies were purchased from Santa Cruz Biotechnology and Amersham Pharmacia Biotech, respectively.

**Cell Lines and Culture Conditions**—Parental DLD1 human colon carcinoma cell line constitutively expresses very low levels of endogenous p21 because of mutations of both alleles of p53 (p53/H11002/H11002) (60). DLD1 cell lines containing exogenous genes for wild-type (WT) or mutant (PCNA/H11002) p21 proteins tagged with HA epitope were as described (9). The p21PCNA/H11002 mutant contains amino acid substitutions (M147A, D149A, and F150A) to abolish the interaction with PCNA specifically. Expression of p21 in these cells is under the tight control of a tetracycline-regulated promoter and can be induced by eliminating tetracycline (Tet) from the culture medium (“Tet-OFF” system). These cell lines were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum (IBL, Maebashi, Japan), 4 mM l-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), and 2 μg/ml tetracycline in a 5% CO2 incubator. Subcloning of these cell lines was repeatedly done in selective medium containing 50 μg/ml hygromycin. Western blotting and flow cytometry was performed to ensure that ~90% of the cells expressed p21 when tetracycline was eliminated. There was no significant difference in the sensitivity to CDDP among parental DLD1, DLD1 p21WT, and DLD1PCNA/H11002 cells.
Experimental Procedures

Western blot analyses as described under the results are demonstrated. Note that essentially the same results. The representative experiments were carried out for three times with equal amounts of cell lysates were used in each immunoprecipitation. The similar experiments indicated antibodies including anti-PCNA, anti-Cdc2, anti-Cdk7, anti-cyclin B1, and PCNA during G2/M phase.

Flow Cytometric Analysis—Expression of p21 and cell cycle analysis were performed by flow cytometry with FACScan (Becton Dickinson, Mountain View, CA) with the program CELLQuest (Becton Dickinson, 61). For detection of p21 protein expression, DLD1 cells (5 × 10^6) were trypsinized, washed with PBS, fixed with 70% ethanol, resuspended in TPBS (PBS containing 0.1% Tween 20), and incubated with fluorescein isothiocyanate-conjugated anti-HA mouse monoclonal antibody (F-7) for 60 min at room temperature in the presence of 0.1% bovine serum albumin (Sigma) and 0.5 μg/ml ribonuclease A (Roche Molecular Biochemicals). The cells were then washed three times with TPBS, stained with propidium iodide (Sigma), and incubated overnight at 4 °C in TPBS containing 0.01% propidium iodide (PI). The cell cycle status of the cells was determined as described previously (24). Briefly, cells (5 × 10^6) were collected, washed twice with cold PBS, and resuspended in Krishan's solution (0.1% sodium citrate, 50 μg/ml PI, 20 μg/ml ribonuclease A, and 0.3% Nonidet P-40) prior to flow cytometric analysis.

Co-immunoprecipitation and Immunoblotting—DLD1 cells (2 × 10^6) were collected, washed twice with PBS, and suspended in 200 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 10 μM NaF, 1 mM Na3VO4, 1 μM okadaic acid, 2 mM diithiothreitol, 0.25% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride) according to the method described previously (62). The lysate was clarified by centrifugation at 15,000 × g for 20 min. The supernatant was collected and incubated with either biotin-conjugated anti-PCNA antibody or anti-HA antibody (for detecting p21) for immunoprecipitation at 4 °C for 1.5 h with gentle rotation. Twenty μl of streptavidin-Sepharose beads (Amersham Pharmacia Biotech) or protein G-Sepharose beads (Amersham Pharmacia Biotech) were added and further incubated for 1 h. The beads were washed 5 times with 1 ml of lysis buffer. Antibody-bound complexes were eluted by boiling in 2× Laemmli sample buffer and resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech). Membranes were blocked in TPBS including 2% non-fat milk for 4 °C overnight, probed with various primary antibodies for 1 h at room temperature, washed three times with TPBS, and probed with the secondary antibody for 40 min at room temperature. The immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal; Pierce).

RESULTS

Conditional Expression of Wild-type and Mutant p21 Proteins in p53-deficient Human Colon Cancer Cell—Repeated subcloning of cells, DLD1 p21WT and DLD1 p21PCNA-, was carried out to enrich cell lines conditionally expressing wild-type or mutant (PCNA−; in which the PCNA-interacting amino acid residues were mutated) p21, respectively. As shown in Fig. 1, A and B, levels of p21 protein expression in these cells were virtually null in the presence of tetracycline but became readily detectable when tetracycline was eliminated from the culture medium. In Fig. 1B, cells were fixed and p21 expression was examined by flow cytometry. Upon induction, p21 protein expression was detected in 94% of DLD1 p21WT and in 87% of DLD1 p21PCNA− cells. Fig. 1C demonstrates the effects of the p21 expression on cell
p21 Binding to PCNA at G₂/M Checkpoint

**Fig. 4. Alternative binding of PCNA to p21 and Cdc25C during G₂/M progression.** Similar co-immunoprecipitation and Western blot (WB) determination was performed with DLD1 p21ΔWT (left panel) and DLD1 p21ΔPCNA (right panel) after the treatment with CDDP as in Fig. 3. The cell lysate was immunoprecipitated with anti-PCNA antibody, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to a membrane. Western blot determination of the PCNA precipitates was performed with antibodies to p21 (HA), Cdc25C, cyclin H, Cdk7, and PCNA. Equal amounts of cell lysates were used in each immunoprecipitation. The similar experiments were carried out for three times with essentially the same results. The representative data are demonstrated here.

proliferation and cell cycle progression by flow cytometry. In the absence of p21 expression, the cell cycle distribution was similar for both cell lines. For example, in DLD1p21ΔWT, 49.3, 15.0, and 35.7% of cells were in G₁, S, and G₂ phases, respectively (Fig. 1C). When p21ΔWT expression was induced in DLD1 cells, a striking decrease in the number of cells in S phase (1.8%) was observed, whereas the majority of cells accumulated at G₁ (39.6%) or G₂/M (58.6%). On the other hand, p21ΔPCNA expression did not significantly change the cell distribution (Fig. 1C, lower panel). These findings confirmed those by Cayrol et al. (9) that p21 was involved in the cell cycle arrest at G₁ and G₂/M through binding to PCNA.

**Requirement of Wild-type p21 in the G₂ DNA Damage Checkpoint—**To examine the effects of p21 expression on the DNA damage-induced G₂ cell cycle arrest, DLD1 p21ΔWT and DLD1 p21ΔPCNA cells were treated with CDDP (12 μg/mL) for 1 h, and p21 proteins were induced by removing Tet, and the cell cycle analysis was carried out by flow cytometry. As shown in Fig. 2A, because most of the cells were accumulated at G₂/M 48–72 h after the DNA damage induced by CDDP, we induced p21 expression by Tet withdrawal (“–Tet”) at 48 h after the CDDP treatment. Cells not expressing p21 or expressing p21 mutant progressed into G₁, and significant numbers of cells underwent cell death (detected as cells in sub-G₁). Fig. 2B indicates that 48 h after the DNA damage ~74% of cells expressing p21ΔWT entered G₂/M, and these cells remained in G₂/M at 120 h. On the other hand, cells not expressing p21 or expressing p21 mutant immediately progressed into G₁. For example, although ~87% of DLD1 cells expressing p21ΔPCNA entered G₂/M at 48 h after the DNA damage, only 50% of the cells were detected at G₂/M at 120 h. Similar observations were obtained with cells not expressing p21. These observations indicate that p21 is involved in the DNA damage-induced G₂ cell cycle arrest and that the ability of p21 to bind PCNA is crucial.

**Binding of p21ΔWT to the PCNA-Cdc2-Cyclin B1 Complex in the DNA Damage G₂ Checkpoint—**It is known that p21 not only inhibits Cdk4/cyclin D1 and Cdk6/cyclin D2 in G₁ checkpoint but also inhibits Cdc2/cyclin B1 during the G₂/M transition at least in vitro (63). We then examined if p21 associates with PCNA at the G₂/M transition together with Cdc2/cyclin B1. In Fig. 3, DLD1 cells were treated with CDDP for 1 h, and expression of either wild type (p21ΔWT) or mutant (p21ΔPCNA) was induced at 48 h after the DNA damage. The interaction of p21 with the Cdc2-cyclin B1 complex and PCNA at the DNA damage G₂ checkpoint was examined by immunoprecipitation (with anti-HA antibody detecting p21ΔWT and p21ΔPCNA) followed by Western blotting (with antibodies to PCNA, cyclin B1, and Cdc2).

As demonstrated in Fig. 3B (left panel), p21ΔWT coimmunoprecipitated PCNA, Cdc2, and cyclin B1 when cells were arrested at G₂/M transition due to CDDP-induced DNA damage, indicating the formation of p21ΔWT-PCNA-Cdc2-cyclin B1 complex in cells arrested at G₂/M. Interestingly, although Cdc2-cyclin B1 complex interacts with p21ΔPCNA at the G₂/M transition (72 h after DNA damage) (Fig. 3, right panel), cells could not maintain the G₂/M arrest (Fig. 2B). In these cells, cyclin B1 was proteolytically degraded and cells entered M and then progressed to G₁ (120 h after DNA damage) (Fig. 3B, right panel). These findings suggest that in order for p21 to induce the cell cycle arrest at G₂ in response to DNA damage, the interaction of p21 with PCNA is crucial. Because p21 was previously shown to inhibit CAK-mediated Cdc2 phosphorylation and promote cell cycle arrest at G₂/M (10), we also examined the interaction of p21 with cyclin H and Cdk7 (CAK). However, neither cyclin H nor Cdk7 was coimmunoprecipitated with p21 (Fig. 3B).

**Involvement of p21ΔWT in G₂/M Checkpoint, Alternative Binding of PCNA to p21ΔWT and Cdc25C—**During the cell cycle interphase, Wee1HU inactivates Cdc2-cyclin B1 complex by phosphorylation of Cdc2 at Tyr-15 (64), which is subsequently activated by Cdc25 through dephosphorylation at Tyr-15 when cells enter mitosis (65). We thus hypothesized that, in human cells, the Cdc25C-mediated activation of Cdc2/cyclin B1 would occur at the nascent DNA following the completion of DNA synthesis or DNA repair, and actions of p21 and Cdc25C would be exclusive. We then examined whether Cdc25C associates with PCNA in the absence of p21, and the PCNA binding of p21 and that of Cdc25C are mutually exclusive (Fig. 4). This pos-
sibility was also prompted by a previous report by Saha et al. (66) that p21 and Cdc25A competitively bind to Cdk2 presum-
ably at the G1-S transition.

In Fig. 4, DNA damage was induced in DLD1 cells, and expression of either wild type (p21WT) or mutant (p21PCNA/H11002) was induced as in Fig. 3. The cell lysate was analyzed for the protein-protein interaction by immunoprecipitation (with anti-PCNA antibody) followed by Western blotting to detect the PCNA-associated p21 and Cdc25C. As demonstrated in Fig. 4 (left panel), Cdc25C was detected in the PCNA complex when p21 was not induced (+ Tet). Cdc25C was coimmunoprecipitated with PCNA in the absence of DNA damage, i.e. in the absence of p21 (data not shown). In the presence of p21WT, PCNA coimmunoprecipitated p21WT but not Cdc25C. However, when p21PCNA was expressed, PCNA coimmunoprecipitated Cdc25C irrespective of the presence of p21PCNA, indicating that p21 and Cdc25C interact with the same region of PCNA.

DISCUSSION

We have explored the role of p21 in the G2 DNA damage checkpoint using p53-deficient cells in which p21WT or p21PCNA was complemented. Our findings clearly indicate the crucial role of p21 in the G2 checkpoint upon DNA damage and that the interaction of p21 with Cdc2-cyclin B1 complex is mediated by PCNA. We assume that the p21-PCNA interaction is probably required for recognition of the repaired DNA. Because the p21-PCNA interaction appeared to be mutually exclusive with the Cdc25C-PCNA interaction at G2/M transition, p21 may prevent the incorporation of Cdc25C into the Cdc2-cyclin B1 complex and thus induce cell cycle arrest at G2. In fact, the formation of ternary complex involving p21, PCNA, and Cdc2/cyclin B1 at G2 was previously demonstrated (7, 19, 63). In addition, Dulic et al. (8) and Medema et al. (67) independently showed the formation of p21-Cdc2-cyclin B1 complex and inhibition of Cdc2 kinase activity at G2. Moreover, we observed that cells failed to arrest at G2/M because of the absence of functional p21 and underwent cell death (Fig. 2). In agreement with this finding, Bunz et al. (5) demonstrated with cells defective for p53 or p21 genes that γ-irradiation induced cell death associated with the lack of cytokinesis after entering into M phase, again indicating the crucial importance of p21 for the maintenance of the G2/M arrest.

Expression of p21 is transcriptionally regulated by p53 upon DNA damage or cellular senescence and is known to induce cell cycle arrest at G2 as well as at G1 by inhibiting Cdc2 (4, 68, 69).
In addition to the binding with Cdc2-cyclin B1 complex, p21 is also shown to bind PCNA (70). PCNA acts as an auxiliary factor for DNA polymerase δ and stimulates DNA replication (71). It was shown that p21 inhibited PCNA-dependent DNA replication in vitro by binding to PCNA through its PCNA-interacting region (11, 12, 72, 73). We found that DLD1, expressing a mutant p21 defective for the interaction with PCNA, could not arrest at G2/M checkpoint even after DNA damage (Fig. 2). Thus, it is possible that, through interacting with PCNA, p21 inhibits DNA synthesis and maintains G2/M arrest.

These findings also suggest an important role for PCNA as a platform for the interaction of various cell cycle regulator proteins that occur adjacent to the nascent DNA or the repaired DNA. Among these proteins, p21, Fen1 (flap endonuclease 1), and xeroderma pigmentosum G are known to contain similar sequences (called PIP box) that interact with PCNA (74). It was shown that Fen1 and p21 compete for binding to the same site of PCNA (75). Similarly, xeroderma pigmentosum G and p21 were shown to compete for the PCNA binding at least in vitro (76). We also found a similar PCNA-binding motif in Cdc25C (Fig. 5A). It is thus conceivable that competitive binding among PCNA-interacting proteins plays an important role in the coordinated DNA replication and repair. Although further studies are needed, it is likely that p21 inhibits cell cycle progression to mitosis by regulating the Cdc25C interaction with the Cdc2/cyclin B1 (Fig. 5B). Considering that fact that PCNA forms a stable homotrimer when it binds to DNA upon DNA synthesis and possibly DNA repair, it remains to be clarified whether these protein-protein interactions with PCNA can occur concomitantly to some extent or are mutually exclusive.

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