Mammalian DNA polymerase (Pol) δ is essential for DNA replication. It consists of four subunits, p125, p50, p68, and p12. We report the discovery that the p12 subunit is rapidly degraded in cultured human cells by DNA damage or replication stress brought about by treatments with UV, methyl methanesulfonate, hydroxyurea, and aphidicolin. The degradation of p12 is due to an accelerated rate of proteolysis that is inhibited by the proteasome inhibitors, MG132 and lactacystin. UV treatment converts Pol δ in vivo to the three-subunit form lacking p12. This was demonstrated by its isolation using immunoaffinity chromatography. The three-subunit enzyme retains activity on poly(dA)/oligo(dT) templates but is impaired in its ability to extend singly primed M13 templates, clearly indicating that its in vivo functions are likely to be compromised. This transformation of Pol δ by modification of its quaternary structure is reversible in vitro by the addition of the p12 subunit and could represent a novel in vivo mechanism for the modulation of Pol δ function. UV and hydroxyurea-triggered p12 degradation is blocked in ATR−/− cells but not in ATM−/− cells, thereby demonstrating that p12 degradation is regulated by ATR, the apical kinase that regulates the damage response in S-phase. These findings reveal a novel addition to the cellular repertoire of DNA damage responses that also impacts our understanding of the role of Pol δ in both DNA replication and DNA repair.

Three eukaryotic DNA polymerases, α, δ, and ε, are involved in chromosomal DNA replication (1, 2). Pol δ (δ3) and Pol ε possess proofreading 3′ to 5′ exonuclease activities, which allows them to replicate DNA with high fidelity (1, 2). Mammalian Pol δ consists of a tightly associated dimer of the 125-kDa catalytic subunit and p50 (4, 5) that is associated with the p68 (6–8) and p12 (9) subunits. The current model for DNA synthesis at the replication fork is that Pol α/primase synthesizes RNA primers plus short stretches of DNA. These are then elongated to Okazaki fragments of ~200 nucleotides by Pol δ on the lagging strand of the replication fork (1, 2). Leading strand synthesis requires highly processive synthesis and was originally thought to require Pol δ, since the latter was able to perform the replication of the SV40 genome in an in vitro system (10). The role of Pol ε has been extensively studied in yeast, where it is essential for viability in both Saccharomyces cerevisiae and Schizosaccharomyces pombe (1, 2). However, it is the C-terminal checkpoint domain and not the N-terminal catalytic domain that is essential (i.e. Pol ε activity per se is dispensable, and it has been suggested that Pol δ activity may be able to replace Pol ε activity) (11, 12). In mammalian cells, it has been found that although Pol δ alone can replicate SV40 DNA replication, both Pol δ and Pol ε are involved in cellular DNA replication (13). Localization and cross-linking studies suggest that Pol δ and Pol ε may function independently of each other and that their involvement in chromosomal replication might be temporally different during S-phase progression (14, 15). Their respective roles in leading and lagging strand synthesis are still uncertain, although it has been proposed that Pol δ and Pol ε function specifically at the lagging and leading strands of the replication fork, respectively (2). In addition to its functions in chromosomal DNA replication, Pol δ is involved in DNA repair as a gap filling enzyme (2) and is recruited to sites of DNA damage (16, 17).

Pol δ requires the DNA sliding clamp PCNA for processive synthesis (1, 2). The p125, p68, and p12 subunits interact with PCNA (18), and p12 and p68 are each required for optimal human Pol δ activity (18, 19). p12 plays a role in stabilizing the Pol δ holoenzyme, and its interaction with PCNA also serves to stabilize the Pol δ-PCNA complex (18). In this study, we show that DNA damage triggers the rapid degradation of p12, with the concomitant conversion of Pol δ to a trimer with altered properties. This observation is of significance, since this may impact the cellular functioning of Pol δ.

In addition, we show that p12 depletion by UV and hydroxyurea (HU) are dependent on the ATR kinase that regulates the DNA damage response in the S-phase but not on ATM. ATM/Chk2, which is activated by double-stranded DNA breaks (20), and ATR/Chk1 (21–23) are the apical kinases in extensive and overlapping signaling networks, which are activated by DNA damage and which regulate the balance between DNA repair and cell death. DNA damage during S-phase elicits an ATR-dependent response that inhibits the G1 to S transition and slows S-phase progression by the transient inhibition of DNA synthesis, in order to allow DNA repair to take place (21–23). ATR is activated by agents that are genotoxic or that inhibit DNA synthesis (e.g. UV and methyl methanesulfonate (MMS),

A Novel DNA Damage Response

RAPID DEGRADATION OF THE p12 SUBUNIT OF DNA POLYMERASE δ

Received for publication, November 7, 2006, and in revised form, February 16, 2007. Published, JBC Papers in Press, February 21, 2007, DOI 10.1074/jbc.M610356200

Sufang Zhang, Yajing Zhou, Sandra Trusa, Xiao Meng, Ernest Y. C. Lee, and Marietta Y. W. T. Lee

From the Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York 10595

*This work was supported by National Institutes of Health Grant GM31973 and Philip Morris USA Inc., Philip Morris International. 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.

†To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595. Tel.: 914-594-4070; Fax: 914-594-4058; E-mail: Marietta_Lee@NYMC.edu.

‡The abbreviations used are: Pol, DNA polymerase; ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia mutated and RAD3-related; RPA, replication protein A; RFC, replication factor C; PCNA, proliferating cell nuclear antigen; Chk1-pS345, Chk1-phosphoserine 345; MMS, methyl methanesulfonate; HU, hydroxyurea; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptidase ligase; DTT, dithiothreitol.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Rapid Degradation of p12 by DNA Damage

H1, which depletes dNTP pools, and aphidicolin, which inhibits the replication DNA polymerases (24, 25). The stalling of replication forks by these treatments leads to the generation of aberrant DNA structures containing single-stranded DNA arising from replication fork collapse, inappropriate homologous recombination (26), or the uncoupling of the replication helicase from Pol α (27). ATR/Chk1 is then activated by its recruitment to RPA-single-stranded DNA, leading to the down-regulation of the Cdc25 phosphatases and the cyclin E/A-cyclin-dependent kinases, so that the entry from G₂ to S-phase is inhibited (21–23). ATR/Chk1 also slows S-phase progression by the inhibition of DNA synthesis, largely by inhibition of the initiation of replication origins via Cdc45, which is required for loading of replication polymerases to the prereplication complex (1, 28).

Our findings that p12 is down-regulated by DNA damage with the concomitant conversion of Pol δ to a trimeric form and that this is under the control of the ATR signaling pathway support the view that the degradation of p12 is a novel cellular response to DNA damage.

MATERIALS AND METHODS

Cell Culture—HeLa, HEK293T cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Human lung carcinoma H1299 and A549 cells (ATCC) were maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum; GM00637 cells (ATM−/−) and GM09607 (ATM−/−) cells obtained from the Coriell Cell Repository and grown in EEMEM (minimum essential medium (Eagle) in Earle balanced salt solution with 2 mM l-glutamine) medium supplemented with 10% fetal bovine serum; HCT116 and ATRflox/− cells (ATCC) were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. One of the ATR alleles in the ATRflox/− cell line is disrupted so that the ATRflox/− cell is ATR−/−. The second allele of ATR has had one of its exons placed under the control of the ATR signaling pathway (21). The cells were grown in growth medium containing 50 μg/ml geneticin, 10 μg/ml cycloheximide, and 1 μg/ml 5-bromodeoxyuridine (BrdU) to select for the Cre-recombinase (29). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum; one of the ATR alleles in the ATRflox/− cell line is disrupted so that the ATRflox/− cell line is ATR−/−. The second allele of ATR has had one of its exons placed between two lox sites and can be disrupted by infection with adenovirus expressing the CRE recombinase to generate the ATR−/− phenotype (21). The cells were grown in growth medium containing 50 multiplicities of infection of Cre-expressing adenovirus (Ad-CMV-Cre; Vector Biolabs, Philadelphia, PA) for 48 h and then treated with UV. H38-5 and ts20TG⁸ mouse cells derived from BALB/c 3T3 A31 were obtained from Dr. Harvey Ozer; ts20TG⁸ has a temperature-sensitive E1 ubiquitin-activating enzyme, and H38-5 is the corrected cell line (29). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 35 °C and shifted to the restrictive temperature of 39 °C for 16–20 h before treatment with UV.

Genotoxic and Inhibitor Treatments—Asynchronous cell cultures were grown to ∼80% confluence. For UV treatment, the culture medium was removed from the cells and irradiated using a UVLMS-38EL series 3 UV lamp (UV-C, 254 nm). Calibration of the UV source was performed with a UV radiometer (UVP, Inc.). Fresh medium was added after treatment, and the dishes were returned to the incubator. Cells were analyzed at different time periods after UV treatment. HU (0.5 mM) was added directly to the culture medium to give final concentrations of 2–8 mM, and the cells were analyzed at different time periods of incubation. Aphidicolin (Sigma) was dissolved in anhydrous Me₂SO and added to the culture medium to give a final concentration of 120 mM. MMS, dissolved in anhydrous Me₂SO (Sigma), was added to the culture medium, and cells were exposed for the stated time periods, following which the cells were transferred to fresh medium and analyzed at different times after treatment. Caffeine (Sigma), MG132, lactacystin (Calbiochem), and UCN-01 (kindly provided by Sally Hausman, NCI, National Institutes of Health) were dissolved in anhydrous Me₂SO and added to the culture medium 30 min before genotoxic treatments.

Antibodies—A rabbit polyclonal antibody against His-p12 was generated. This antibody was affinity-purified using immobilized p12 and was used for all of the studies reported unless otherwise stated. A rabbit polyclonal antibody against a p12 peptide (18) was also tested and gave similar results to the polyclonal antibody in that p12 depletion was observed after UV treatment. The latter finding makes it unlikely that the loss of p12 observed by Western blotting is an artifact due to a masking of a p12 epitope. Antibodies against the p12⁵, p50, and p68 subunits of Pol δ were obtained as previously described (18). PCNA and ATR antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Chk1-pS345 antibody was obtained from Cell Signal Technology, Inc. Antibody against β-actin was obtained from Sigma.

Western Blotting—Cells were harvested and collected by centrifugation (1000 rpm) for 5 min and lysed by the addition of lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% (v/v) Nonidet P-40, protease inhibitor mixture (Sigma), 50 μg/ml phenylmethylsulfonylate, 0.2 mM sodium orthovanadate). The suspended cells were vortexed and centrifuged at 10,000 × g for 10 min. Control experiments confirmed that all of the Pol δ subunits were released into the supernatant. Supernatants were subjected to SDS-PAGE on 12.5% polyacrylamide gels, following which the separated proteins were transferred to nitrocellulose. Protein loading was maintained at 60 μg/lane. Western blotting was performed with antibodies against p12, p12⁵, p50, and p68 as previously described (18, 30).

Immunoaffinity Purification of Pol δ—HEK293T cells were grown in 150 × 25-mm plates (Corning Glass). Cells from 80 plates (2 × 10⁶ cells) were used. Half of the plates were treated with UVC (20 J/m²) and harvested 4 h later; the other half was harvested with no treatment. The pelleted cells were lysed in 20 ml of lysis buffer (20 mM Tris-HCl, pH 7.8, 10% glycerol, protease inhibitor mixture (Sigma), 1 mM phenylmethylsulfonylate, 1 mM EDTA, 1 mM EGTA, 1 mM MgCl₂) and disrupted by passage through a French press. The suspension was centrifuged, and the supernatant was diluted to 40 ml with lysis buffer. The protein content was determined, and equal amounts of total protein were used for the following chromatography. Two columns of identical size, each containing 5 ml of anti-p125-agarose beads (18, 30, 31) were used for the chromatography of the lysates of the UV-treated and untreated cells. The columns were run at the same time under the same conditions. The columns were washed with 10 column volumes
of 0.1 M NaCl in TGGE buffer (40 mM Tris-HCl, pH 7.8, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA) and then eluted with 30% ethylene glycol, 0.4 M NaCl in TGGE buffer. Fractions of 0.3 ml each were collected. Chromatography was performed at 4 °C. Assays of the column fractions for Pol δ activity as well as other analyses were performed in parallel with minimum delay. Western blots of any given protein from the two columns were developed in the same solutions and visualized on the same x-ray film to provide comparable staining. Recombinant Pol δ and the trimer lacking p12 were produced by expression in Sf9 cells and were purified to near homogeneity by methods previously described (18).

Assay for Pol δ Activity—The standard assay for Pol δ activity was performed using poly(dA)/oligo(dT) as described previously (18, 30, 31). Standard reactions for the poly(dA)/oligo(dT) assay contained 0.25 OD units/ml poly(dA)4000/oligo(dT)50 (Supertechs, Bethesda, MD), 200 μg/ml bovine serum albumin, 5% glycerol, 10 mM MgCl₂, 25 mM HEPES, pH 6.0, 100 cpm/μmol [³²P]TTP, and 1.0 units of Pol δ in the absence or presence of 100 ng of PCNA in a total volume of 30 μl. One unit of Pol δ activity catalyzes the incorporation of 1 nmol of dTMP/h of TEMP/h at 37 °C. Assays using singly primed M13 DNA as the template were performed as previously described (18). M13mp18 DNA (New England Biolabs, Ipswich, MA) was primed with a 20-mer oligonucleotide (5'-CTAGAGGATCCCCCGTTA-CC'-3') complementary to nucleotides 6262–6243. The standard reaction contained 40 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 10 mM MgCl₂, 0.5 mM ATP, 50 mM NaCl, 250 μM each of dTTP, dCTP, and dGTP, 25 μM dATP, 3 μCi of [α-³²P]dATP, 100 ng of primed M13 template, 80 ng of RFC, 200 ng of RPA, and variable amounts of PCNA in a 30-μl reaction volume. The reaction mixtures were incubated at 37 °C for 30 min and were terminated by the addition of 20 mM EDTA. The reaction products were run on 1.5% alkaline agarose gels at 50 V for 2.5 h. The gels were visualized with a PhosphorImager.

Reconstitution of Pol δ from Pol δ<sub>3-p12</sub> with Recombinant p12—Pol δ<sub>3-p12</sub> was reincubated with recombinant p12 or His-tagged p12 at a final concentration of 3.3 or 6.6 μg/ml at 4 °C for 1 h before assay for Pol δ activity. Recombinant p12 was expressed as glutathione S-transferase-tagged p12 in the pGEX5X-3 vector (GE Healthcare), and purified on glutathione-Sepharose. p12 was then released by proteolysis with factor Xa, and the glutathione S-transferase was removed with glutathione-Sepharose. His-tagged p12 was expressed in Escherichia coli and purified by chromatography on Ni²⁺-nitrilotriacetic acid beads as previously described (18).

In Vivo Ubiquitination Assay—The human p12 coding region was cloned into the mammalian expression vector pCDNA4/HisMaxC (Invitrogen) between the BamHI and EcoRI sites; the resultant plasmid expressed N-terminal His-tagged p12. The human ubiquitin coding region with a FLAG tag at the N terminus was cloned as a BamHI/EcoRI fragment into pCDNA3 (Amersham Biosciences). HEK293T cells were co-transfected with His-p12 together with FLAG-tagged ubiquitin using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h, treatment with MG132 was carried out at a final concentration of 10 μM for 2 h prior to treatment with UVC (10 J/m²), followed by further incubation for 1 h before harvesting. Transfected cells in a 10-cm dish were washed twice with phosphate-buffered saline and then harvested directly into 1 ml of lysis buffer (8 M urea, 0.1 M NaHPO₄, 0.01 M Tris-HCl, pH 8.0, 10 mM dithiothreitol) and sonicated. Protein concentration was measured with a protein assay kit (Bio-Rad). One mg of total protein extract was incubated with 80 μl of a 50% slurry of nickel-agarose beads (Ni²⁺-nitrilotriacetic acid beads; Qiagen) for 1.5 h in a rotator at 4 °C. The beads were washed 10 times, twice with 1 ml of each of the following buffers to minimize deubiquitination (32): 1) 8 M urea, 0.1 M NaHPO₄, 0.01 M Tris-HCl, pH 8.0, 10 mM DTT; 2) 6 M guanidine HCl, 0.1 M NaHPO₄, 0.01 M Tris-HCl, pH 8.0, 10 mM DTT, 5 mM imidazole; 3) 8 M urea, 0.1 M NaHPO₄, 0.01 M Tris-HCl, pH 8.0, 10 mM DTT; 0.1% Triton X-100; 4) 8 M urea, 0.1 M NaHPO₄, 0.01 M Tris-HCl, pH 6.3, 10 mM DTT, 0.1% Triton X-100; 5) 8 M urea, 0.1 M NaHPO₄, 0.01 M Tris-HCl, pH 6.3, and 10 mM DTT. After these washes, bound proteins were eluted with 20 μl of 6X SDS sample loading buffer (Amersham Biosciences) mixed with 80 μl of 250 mM imidazole, 10 mM DTT. Proteins were separated by SDS-gel electrophoresis, and p12 was detected by immunoblotting with anti-p12 monoclonal antibody (Abnova Taiwan Corp., Taipei, Taiwan); the blots were then stripped and reblotted with anti-FLAG monoclonal antibody (M2; Sigma). For these experiments, 6% gels were used over a bottom layer of 12% polyacrylamide in order to retain the p12 while allowing the separation of the high molecular weight polyubiquitinated products.

RESULTS

The p12 Subunit of Pol δ Is Depleted by UV Treatment of Cells—Exposure of cultured human cells to UV results in a rapid and specific depletion of the p12 subunit of Pol δ. p12 levels were depleted below detection levels by 6 h after exposure of HeLa cells to 10–40 J/m² UVC and recovered by 24 h (Fig. 1A). The other three subunits of Pol δ and PCNA were relatively unaffected (Fig. 1A). At lower UV doses, between 1 and 10 J/m² (Fig. 1B), p12 levels were reduced to near completion by 5, 4, and 10 J/m² UV in HeLa, HEK293T, and HCT116 cells, respectively, 4 h after treatment. p12 depletion was time-dependent, requiring 1 h for HeLa cells and 4 h for HCT116 cells after treatment with 10 J/m² UV (Fig. 1C). Because ATR/Chk1 signaling is activated by UV damage (21–23), a Western blot for Chk1-pS345 was also performed (33) (Fig. 1, B and C).

We noted that HCT116 cells were more resistant than HeLa cells or HEK293T cells to p12 depletion by UV (Fig. 1, B and C). HCT116 cells differ in that they are p53-competent, whereas the other two are not. We tested the idea that this difference is due to p53 status in two human lung cell lines, H1299 (p53 null) and A549 (p53 wild type). p12 in the H1299 cell line was largely depleted at a UV dose of 1 J/m² but required 20 J/m² in A549 cells (Fig. 1D). Similar observations were made with other p53 competent cells (not shown). The reason for the slower rate of p12 degradation is unknown but could reflect the ability of p53 to stabilize stalled replication forks and inhibit the generation of aberrant DNA structures (34, 35).
p12 Is Degraded by Treatment of Cells with MMS, HU, or Aphidicolin—Next, it was determined if the degradation of p12 occurs after other genotoxic treatments that are known to trigger the intra-S-phase checkpoint via ATR/Chk1 activation. p12 was degraded after MMS treatment in HeLa and HEK293T cells in a dose-dependent manner (Fig. 2A), with concomitant phosphorylation of Chk1-Ser-345. p12 degradation after MMS treatment was time-dependent (Fig. 2B), and depletion was reached by 2 h in HeLa and by 30 min in HEK293T cells. HCT116 cells were much more resistant, and depletion was only observed after 9 h. The sensitivity of H1299 (p53 null) and A549 (p53 wild type) cells to MMS was examined. p12 was depleted by MMS in H1299 (p53 null) cells by 1 h after treatment but was only partially degraded after 12 h in A549 (p53 wild type) cells (Fig. 2C), similar to what was observed for UV treatment (Fig. 1D).

Hydroxyurea (2 mM) caused the degradation of p12 in HeLa and HEK293T cells (Fig. 3A), but much more slowly by comparison with UV, nearing completion after 20 h. The depletion of p12 was correlated with the phosphorylation of Chk1 (Fig. 3A). Aphidicolin treatment of H1299 cells caused the depletion of p12 after 20 h (Fig. 3B).

p12 Depletion Is Due to an Accelerated Rate of Proteolysis That Requires Proteasome Activity—The degradation of p12 by UV in the presence of the protein synthesis inhibitor, cycloheximide, was examined (Fig. 4A). The half-life of p12 was ~11 h, but it was reduced by ~30-fold to 24 min after UV treatment (Fig. 4B). Thus, the depletion of p12 is due to an accelerated rate of proteolysis. Since many cellular processes are regulated via the polyubiquitination of proteins that targets them to the 26S proteasomes for their degradation (36, 37), evidence was sought to determine whether this is the case for p12. First, in order to determine whether the UV-dependent proteolysis of p12 is mediated by proteasomes, the effects of a commonly used inhibitor of proteasome activity, MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) on p12 degradation were examined. MG132 blocked the degradation of p12 after UV, HU, and aphidicolin treatments (Fig. 5A). Since MG132 also inhibits calpain and cathepsin B (38), we also determined the effects of lactacystin. Lactacystin, a highly specific inhibitor of proteasome activity with no known actions on other proteases that have been tested (39), also prevented p12 depletion after UV treatment of H1299.
Rapid Degradation of p12 by DNA Damage

FIGURE 3. Effects of HU and aphidicolin on p12 levels. A, hydroxyurea (2 mM) was added to the culture medium for HeLa and HEK293T cells. Cells were harvested and analyzed for p12 and Chk1-pS345 by Western blotting at the indicated times. B, aphidicolin (120 nM) was added to the culture medium for H1299 cells. Cells were harvested at the indicated times after the addition of aphidicolin and analyzed for p12 by Western blotting.

FIGURE 4. p12 depletion is due to an accelerated rate of proteolysis. A, HeLa cells were incubated with 10 μg/ml cycloheximide (CHX) and then treated with UV (10 J/m²) and blotted for p12 levels at the indicated times. B, p12 levels in A were estimated by scanning, and the relative levels after normalization with the actin loading control were plotted against time. Solid squares, untreated cells; solid circles, UV-treated cells.

and A549 cells (Fig. 5B). These results show that p12 degradation requires proteasomal protease activity.

It should be noted that both MG132 and lactacystin led to the stabilization of p12 levels (i.e. p12 levels are in fact slightly increased over the controls). The stabilization of proteins that are targeted for proteasome degradation by proteasome inhibitors is a common observation (i.e. very little accumulation of polyubiquitinated proteins takes place in vivo) (40, 41), even in the presence of proteasome inhibitors. This is attributable to the rapid in vivo depolymerization of ubiquitin chains by deubiquitinating enzymes (some of which are associated with the 26 S proteasomes) to promote a dynamic process of deubiquitination coupled to protein degradation (42, 43).

UV-dependent p12 Degradation Requires E1 Ubiquitin-activating Enzyme Activity—The enzymatic process for polyubiquitination is a pyramidal arrangement of a single E1 ubiquitin-activating enzyme that is obligatory for the transfer of ubiquitin to the E2 ubiquitin-conjugating enzymes, which in turn cooperate with many more E3 ligases to ubiquitinate a multitude of substrates (36, 37). In order to determine whether ubiquitination is involved in the process of p12 depletion, the effects of UV on p12 levels were examined in the ts20TG⁰ cell line that has a temperature-sensitive E1 ubiquitin-activating enzyme. As a consequence, the ubiquitin pathway can be conditionally suppressed at the restrictive temperature of 39 °C by loss of the single cellular E1 activating enzyme (29), p12 was depleted after treatment with 10 J/m² UV at the permissive temperature of 35 °C in these cells and was resistant in the presence of MG132 as previously shown for other cell lines but was not degraded at the restrictive temperature of 39 °C (Fig. 5C, top). The control experiments performed using H38-5 cells that had been corrected by stable transfection of E1 (Fig. 5C, bottom). The dose dependence of p12 degradation by UV in ts20TG⁰ cells is shown in Fig. 5D. Whereas a nearly complete degradation of p12 was observed at 35 °C at a UV dose of 5 J/m², p12 degradation was prevented even at UV doses as high as 30 J/m² at the restrictive temperature of 39 °C (Fig. 5D, bottom). In addition, it is noted that p12 degradation is blocked at both low and high doses of UV (Fig. 5D), in contrast to the effects observed in ATR−/− cell lines (see below). These results show that p12 degradation by UV requires an active ubiquitin pathway.

In Vivo Assay for the Presence of Polyubiquitinated Forms of p12—Although the findings made above provide evidence that UV-triggered p12 degradation in vivo is mediated by the proteasomes and requires a functional ubiquitination pathway, they do not establish that p12 itself is ubiquitinated. In order to gain information on the issue of whether in vivo ubiquitination takes place, we used the assay involving the cotransfection of FLAG-tagged ubiquitin together with a His-tagged target protein. FLAG-tagged ubiquitin and His-tagged p12 were cotransfected into HEK293T cells before UV treatment in the presence
and absence of MG132. Because of the small fraction of polyubiquitinated products that are formed, the His-tagged p12 was recovered using nickel-agarose beads and extensively washed in strong denaturants to minimize deubiquitination during the processing of the lysates (32). The His-tagged p12 was then analyzed by Western blotting with a p12 monoclonal antibody and then reprobed with anti-FLAG antibody. Preliminary experiments using 12% polyacrylamide showed that the bulk of the products ran at the top of the gel, indicating that they were of high molecular weight. A 6% polyacrylamide gel was therefore used, with a bottom layer of 12% polyacrylamide to retain the His-p12 (Fig. 6). In untreated cells, very little polyubiquitinated products of His-p12 were observed (Fig. 6, lanes 1, left and center), with a slight increase in the presence of MG132 (Fig. 6, lanes 2, left and center). UV-treated cells exhibited a similar pattern as the untreated cells, but in UV-treated cells in the presence of MG132 there was a discernable increase in the amount of polyubiquitinated products. (The apparent band at ~80 kDa does not represent a single species but is probably due to a concentration of proteins at the 6–12% gel interface, since this was not observed on 12% gels.) The appearance of very high molecular weight products is not unusual in ubiquitination reactions, and it is noted that we did not observe low molecular weight intermediates. However, it should be noted that some E2-E3 complexes can catalyze apparently processive ubiquitination reactions by the transfer of preassembled ubiquitin chains (44). As noted above, the low levels of ubiquitination observed are probably due to rapid in vivo deubiquitination, and as yet no cell-permeable inhibitors of deubiquitinating enzymes are available to offset this problem (40).

Pol δ Is Transformed from a Tetramer to a Trimer in Vivo by UV Damage—In order to determine whether the loss of p12 converts Pol δ to an intact three-subunit enzyme or leads to the dissociation of the remaining Pol δ subunits, Pol δ from UV-treated and control HEK293T cells were isolated by immunoprecipitation on anti-p125-agarose beads. (We have used this procedure for the purification of Pol δ from calf thymus (9, 31) and HeLa cells (45) and of Pol δ overexpressed in SF9 cells (18, 30). The experiment was performed three times, and representative data are shown in Fig. 7. Western blot analysis showed that p125, p68, p50, and p12 were present in the bound fractions of the untreated cells as expected, whereas only p125 p68 and p50 were present in the bound fractions in the UV-treated cells (Fig. 7A). The retention of the p68 and 50 subunits in the bound enzyme from the UV-treated cells shows that loss of the p12 subunit does not result in the dissociation of the remaining subunits (i.e. that Pol δ from the UV-treated cells is an intact complex of the p125, p50, and p68 subunits). Since UV treatment does not result in significant loss of the p125 subunit (see Fig. 1), it suggests that most, if not all, of the Pol δ is converted to the three-subunit form in vivo. This three-subunit form of Pol δ will be referred to as Pol $\delta_{3-p12}$ for convenience.

The column fractions were assayed for Pol δ activity using poly(dA)/oligo(dT) as the template-primer (Fig. 7B). The total activity recovered in the peak fractions from the UV-treated cells was 45% of that from the control cells (recoveries of 54 and 85% were obtained in the two other experiments; i.e. an average of 61 ± 21%). These recoveries should approximate the specific activity of Pol $\delta_{3-p12}$ relative to that of Pol δ taken as 100, since comparable amounts of protein were present in the two lysates. This is supported by the reactivation of Pol $\delta_{3-p12}$ activity in the peak fraction to >90% of the activity of the peak fraction from the untreated cells after precubination with p12 (see Fig. 9A). This latter finding also supports the view that most of the Pol δ is converted to Pol $\delta_{3-p12}$ after UV treatment.

The exhibition of a significant level of activity on poly(dA)/oligo(dT) by Pol $\delta_{3-p12}$ was unexpected, since previous studies of the recombinant human Pol δ trimer lacking p12 reported that it has very poor activity in this assay (18, 19). We have reexamined the activity of very highly purified preparations of recombinant Pol δ and the recombinant trimer missing the p12 subunits in this assay (Fig. 7C). The recombinant trimer lacking p12 was comparatively PCNA-insensitive and exhibited a specific activity <2% of that of the tetramer in the presence of PCNA. In previous studies, values of 20% (18) and ~8% (19) have been reported. The reasons for this difference between Pol $\delta_{3-p12}$ produced in vivo and its recombinant counterpart are unknown. The retention of activity by Pol $\delta_{3-p12}$ is significant, since it suggests that it may be active in vivo and may be able to perform lagging strand synthesis, which requires synthesis of short stretches of DNA, or participate in the resynthesis steps of DNA repair in vivo.

Pol $\delta_{3-p12}$ was examined for its ability to perform highly processive synthesis on singly primed M13 DNA (18). This assay contains PCNA, RFC, and RPA. It requires that PCNA be loaded onto the RPA-coated M13 DNA by its clamp loader,
Rapid Degradation of p12 by DNA Damage

**A**

- **Untreated Cells**
  - p125
  - p68
  - p50
  - p12

- **UV-treated Cells**
  - p125
  - p68
  - p50
  - p12

**B**

- **Pol δ Activity (cpm incorporated)**
  - Untreated
  - UV-treated
  - Pol δ
  - PCNA

**C**

- **Δp12 (Pol δ/p12)**
  - Recombinant
  - Native

---

RFC, and the products are examined by alkaline gel electrophoresis to determine the capacity of the enzyme for processive synthesis of the 7-kb M13 DNA. Pol δ3-p12 was defective in this assay, since the products were much smaller by comparison with those of Pol δ, which synthesized products consistent with the length (7 kb) of M13 DNA (Fig. 8A). The accumulation of products of ~3 kb may be due to stalling of Pol δ at sites of secondary structure. In addition, the activity appears significantly lower by comparison of the intensity of incorporation of radioactivity. The poor ability of Pol δ3-p12 to extend the single primer on M13 DNA suggests that it is defective in processive synthesis, which could arise from an altered ability to interact with PCNA or the other replication proteins in the M13 assay or an inability to bypass regions of secondary structure in the template. The behavior of the reconstituted Pol δ3-p12 in this assay is shown in Fig. 8B, and it also is defective in this assay.

Pol δ containing p12 with a mutation in its PCNA binding site exhibited properties similar to those of Pol δ3-p12 (viz. about 60% of the activity of unmodified Pol δ on poly(dA)/oligo(dT) and an impaired ability to carry out processive DNA synthesis on singly primed M13 DNA) (18). These findings indicate the importance of p12 in the interaction of Pol δ with PCNA, which is revealed by a defective behavior in the M13 assay (18). It may be noted that the trimer that lacks p68 is fully active on poly(dA)/oligo(dT) but is also defective in the M13 assay (18).

The addition of recombinant p12 to Pol δ3-p12 led to the restoration of its activity on poly(dA)/oligo(dT) (Fig. 9A). Untagged p12 restored the activity of Pol δ3-p12 to 93% of that of the untreated enzyme, whereas Histagged p12 was slightly less effective. The activity on M13 DNA was also significantly restored by the addition of p12 (Fig. 9B, cf. lane 2 with lanes 3 and 4) but not by Histagged p12 (Fig. 9B, cf. lane 2 with lanes 5 and 6). However, it is noted that the products obtained were in the 3 kb range (i.e. complete restoration of processivity was not found in this experiment). Nevertheless, our data suggest that the removal of p12 may be reversible (i.e. it implies that Pol δ might be reconstituted from Pol δ3-p12 in vivo without the necessity for the resynthesis of the entire Pol δ complex). Thus, the interconversion of Pol δ and Pol δ3-p12 could represent a novel mechanism for the modulation of Pol δ function.

**p12 Degradation Is Dependent on ATR/Chk1**—The question arises as to whether ATR/Chk1 is required for p12 depletion, since both are triggered by DNA damage that results in replication fork stalling. Caffeine, an inhibitor of ATR and ATM in vitro and an inhibitor of cell cycle checkpoints (46), prevented p12 degradation by UV doses as high as 30 J/m2 (Fig. 10A) as well as by HU (Fig. 10B). UCN-01, an inhibitor of Chk1 (47), inhibited p12 degradation with the concurrent inhibition of Chk1 phosphorylation (Fig. 10C). p12 degradation was not completely prevented, but there nevertheless was an inverse correlation between p12 and Chk1 phosphorylation. The depletion of p12 by HU treatment was completely blocked by UCN-01 (Fig. 10D).

We examined the effects of UV on p12 in ATR-deficient cells using the ATR<sup>−/−</sup> cell line (see “Materials and Methods”) (21). p12 degradation was completely blocked in ATR<sup>−/−</sup> cells at a
UV dose of 5 J/m², whereas p12 was depleted by 2 h in the ATR⁺/⁺ and ATR⁻/⁻ cells (Fig. 11A, top). At 15 J/m², p12 was depleted by 1–2 h in the ATR⁺/⁺ and ATR⁻/⁻ cells but was not completely blocked in ATR⁻/⁻ cells, although there was a delay in the depletion of p12 to 5 h (Fig. 11A, bottom). Thus, p12 degradation is dependent on ATR/Chk1 at low but not high UV doses. Controls showed that Chk1 was not phosphorylated in the ATR⁻/⁻ cells (Fig. 11B) at either UV dose. At high UV, p12 depletion was not prevented despite the absence of Chk1 phosphorylation, indicating that an ATR/Chk1-independent signaling pathway comes into play.

We then examined the effects of HU on p12 levels in ATR⁻/⁻ cells (Fig. 11C). Although p12 was depleted in the ATR⁺/⁻ cells by 16 h, this was blocked in the ATR⁻/⁻ cells. These findings are consistent with those observed with UCN-01 (Fig. 10D) and support the view that p12 depletion by HU is ATR/Chk1-dependent. Controls showed that ATR protein was absent in the ATR⁻/⁻ cells (Fig. 11D).

In order to determine whether ATM signaling is likely to play a role in p12 degradation, the effects of UV and HU on ATM⁺/+ (GM00637) and ATM⁻/⁻ (GM09607) cell lines were examined (Fig. 11E). p12 depletion was unaffected in ATM⁻/⁻ cells after a UV dose of 6 J/m² or by treatment with 4 mM HU. Therefore, p12 degradation may not be ATM-dependent.

**DISCUSSION**

We have rigorously demonstrated that the p12 subunit of Pol δ is rapidly degraded in human cell lines after treatments with UV, MMS, HU, and aphidicolin. The UV-mediated degradation of p12 results in the in vivo conversion of the Pol δ tetramer to the trimer, Pol δ₂₅₂. Pol δ₂₅₂ exhibits ~60% of the activity of its progenitor when assayed using poly(dA)/oligo(dt) but is defective in processive synthesis on M13 templates (i.e. it appears unlikely to be able to function as a leading strand polymerase in vivo). Although it is tempting to consider that p12 depletion may be involved in the inhibition of DNA synthesis that is observed after DNA damage with UV (see below), this may be too simplistic an explanation. It has been reported that p12 silencing in mouse endothelial cells causes a significant decrease in FGF2 (fibroblast growth factor)-stimulated cell growth (48). In these cells, p12 expression is specifically up-regulated by FGF2 but not by other mitogenic stimuli (48). Although the findings that p12 levels can affect cell proliferation are consistent with the hypothesis that this is due to effects on DNA synthesis via the modification of Pol δ, a defect in Pol δ
function in leading strand synthesis would be expected to be lethal. There are several possible explanations for why this is not the case. The first is that Pol δ3-p12 retains its ability to act as a lagging strand polymerase but is not essential for the processional DNA synthesis that occurs at the leading strand. This interpretation supports the proposal that Pol δ functions specifically as a lagging strand polymerase, whereas Pol ε acts specifically as a leading strand polymerase (2). Recent studies of Pol ε-depleted Xenopus oocyte extracts also support an essential function of Pol ε activity in chromosomal DNA replication (49), in contrast to the situation in yeast. The second possibility is that Pol ε can act for Pol δ at the leading strand under certain conditions, similar to the situation in yeast, where it has been proposed that Pol δ takes over the functions of Pol ε, as suggested by the viability of yeast mutants containing Pol ε mutants that are catalytically inactive (11, 12). This interpretation is consistent with a situation in which both Pol δ and Pol ε are involved in leading strand synthesis (13–15). A third possibility is that a novel Pol δ-binding protein(s) could interact with Pol δ3-p12 subsequent to DNA damage and functionally compensate for the loss of p12. Several Pol δ-binding proteins have been identified, including PDIP38 (polymerase δ-interacting protein) and PDIP46 (45), the Werner helicase (50), and Werner heli-
Our findings that p12 degradation is dependent on ATR/Chk1 at low UV doses but is independent of ATR/Chk1 at high doses, suggest a second signaling pathway. Low doses of UV (<1 J/m²) are thought to have very low cytotoxicity, whereas doses from 5 to 10 J/m² are cytotoxic (56). The low ability to interact with DNA replication, initiation complex, or whether this involves modifications of its intrinsic activity or its cellular consequences may shed new light on the mechanisms involved in the intra-S-phase checkpoint.

Rapid Degradation of p12 by DNA Damage

1. Takeda, D. Y., and Dutta, A. (2005) Oncogene 24, 2827–2843
2. Garg, P., and Burgers, P. M. (2005) Crit. Rev. Biochem. Mol. Biol. 40, 115–128
3. Byrnes, J. J., Downey, K. M., Black, V. L., and So, A. G. (1976) Biochemistry 15, 2817–2823
4. Lee, M. Y., Tan, C. K., Downey, K. M., and So, A. G. (1981) Prog. Nucleic Acid Res. Mol. Biol. 26, 83–96
5. Lee, M. Y., Tan, C. K., Downey, K. M., and So, A. G. (1984) Biochemistry 23, 1906–1913
6. Hughes, P., Stratner, I., Ducoux, M., Piard, K., and Baldacci, G. (1999) Nucleic Acids Res. 27, 2108–2114
7. Mo, J., Liu, L., Leon, A., Mazloum, N., and Lee, M. Y. (2000) Biochemistry 39, 7245–7254
8. Shikata, K., Ohta, S., Yamada, K., Obuse, C., Yoshikawa, H., and Tsurui, T. (2001) J. Biochem. (Tokyo) 129, 699–708
9. Liu, L., Mo, J., Rodriguez-Belmonte, E. M., and Lee, M. Y. (2000) J. Biol. Chem. 275, 18739–18744
10. Waga, S., and Stillman, B. (1994) Nature 369, 207–212
11. Feng, W., and D’Urso, G. (2001) Mol. Cell. Biol. 21, 4495–4504
12. Kesti, T., Flick, K., Kerenan, S., Syvaoja, J. E., and Wittenberg, C. (1999) Mol. Cell 3, 679–685
13. Zlotkin, T., Kaufmann, G., Jiang, Y., Lee, M. Y., Uitto, L., Syvaoja, J., Dornreiter, I., Fanning, E., and Nethanel, T. (1996) EMBO J. 15, 2298–2305
14. Fuss, J., and Linn, S. (2002) J. Biol. Chem. 277, 8658–8666
15. Rytkonen, A. K., Vaara, M., Nethanel, T., Kaufmann, G., Sormunen, R., Laara, E., Nasheuer, H. P., Rahmeh, A., Lee, M. Y., Syvaoja, J. E., and Pospiech, H. (2006) FEBS J. 273, 2984–3001
16. Jurvansuu, I., Raj, K., Stasiak, A., and Beard, P. (2005) J. Virol. 79, 569–580
17. Perucca, P., Cazzalini, O., Mortewsczitz, O., Necchi, D., Savio, M., Nardo, T., Stivala, L. A., Leonhardt, H., Cardoso, M. C., and Prosperi, E. (2006) J. Cell Sci. 119, 1517–1527
18. Li, H., Xie, B., Zhou, Y., Rahmeh, A., Trusa, S., Zhang, S., Gao, Y., Lee, Y. C. Y., and Lee, M. Y. (2006) J. Biol. Chem. 281, 14748–14755
19. Podust, V. N., Chang, L. S., Ott, R. G., Dianov, L., and Fanning, E. (2002) J. Biol. Chem. 277, 3894–3901
20. Kitagawa, R., and Kastan, M. B. (2005) Cold Spring Harb. Symp. Quant. Biol. 70, 99–109
21. Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001) Science 294, 1713–1716
22. Zou, L., and Elledge, S. J. (2003) Science 300, 1542–1548
23. Petermann, E., and Caldecott, K. W. (2006) Cell Cycle 5, 2203–2209
24. Merrick, C. J., Jackson, D., and Diffley, J. F. (2004) J. Biol. Chem. 279, 2067–2075
25. Hammond, E. M., Green, S. L., and Giaccia, A. J. (2003) Mutat. Res. 532, 205–213
26. Andreassen, R. R., Ho, G. P., and D’Andrea, A. D. (2006) Carcinogenesis 27, 883–892
27. Byun, T. S., Pacek, M., Yee, M. C., Walter, J. C., and Cimprich, K. A. (2005) Genes Dev. 19, 1040–1052
28. Machida, Y., Hamlin, J. L., and Dutta, A. (2005) Cell 123, 13–24
29. Chowdary, D. R., Dermondy, J. I., Jha, K. K., and Ozer, H. L. (1994) Mol. Cell. Biol. 14, 1997–2003
30. Xie, B., Mazloum, N., Liu, L., Rahmeh, A., Li, H., and Lee, M. Y. (2002) Biochemistry 41, 13133–13142
31. Jiang, Y., Zhang, S. J., Wu, S. M., and Lee, M. Y. (1995) Arch. Biochem. Biophys. 320, 297–304
32. Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999) EMBO J. 18, 6455–6461
33. Zhao, H., and Piwnica-Worms, H. (2001) Mol. Cell. Biol. 21, 4129–4139
34. Gatz, S. A., and Wiesmüller, L. (2006) Cell Death Differ. 13, 1003–1016
35. Sengupta, S., and Harris, C. C. (2005) Nat. Rev. Mol. Cell. Biol. 6, 44–55
36. Herrishko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
37. Pickart, C. M., and Cohen, R. E. (2004) Nat. Rev. Mol. Cell Biol. 5, 177–187
38. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761–771
39. Fenteany, G., and Schreiber, S. L. (1998) J. Biol. Chem. 273, 8425–8458
40. Volk, S., Wang, M., and Pickart, C. M. (2005) Methods Enzymol. 399, 3–20
41. Kaiser, P., and Tagwerker, C. (2005) Methods Enzymol. 399, 243–248
42. Amerik, A. Y., and Hochstrasser, M. (2004) Biochem. Biophys. Acta 1695, 189–207
43. Guterman, A., and Glickman, M. H. Curr. Protein Pept. Sci. 5, 201–211
44. Brzovic, P. S., Lissoumov, A., Christensen, D. E., Hoyt, D. W., and Klevit, R. E. (2006) Mol. Cell 21, 873–880
**Rapid Degradation of p12 by DNA Damage**

45. Liu, L., Rodriguez-Belmonte, E. M., Mazloum, N., Xie, B., and Lee, M. Y. (2003) *J. Biol. Chem.* 278, 10041–10047
46. Kaufmann, W. K., Heffernan, T. P., Beaulieu, L. M., Doherty, S., Frank, A. R., Zhou, Y., Bryant, M. F., Zhou, T., Luche, D. D., Nikolaiashvili-Feinberg, N., Simpson, D. A., and Cordeiro-Stone, M. (2003) *Mutat. Res.* 532, 85–102
47. Busby, E. C., Leistritz, D. F., Abraham, R. T., Karnitz, L. M., and Sarkaria, J. N. (2000) *Cancer Res.* 60, 2108–2112
48. Dell’Era, P., Nicoli, S., Peri, G., Nieddu, M., Ennas, M. G., and Presta, M. (2005) *Oncogene* 24, 1117–1121
49. Shikata, K., Sasa-Masuda, T., Okuno, Y., Waga, S., and Sugino, A. (2006) *BMC Biochem.* 7, 21
50. Szekely, A. M., Chen, Y. H., Zhang, C., Oshima, J., and Weissman, S. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 11365–11370
51. Tsurimoto, T., Shinozaki, A., Yano, M., Seki, M., and Enomoto, T. (2005) *Genes Cells* 10, 13–22
52. Lehmann, A. R. (2006) *Exp. Cell Res.* 312, 2673–2676
53. Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004) *Annu. Rev. Biochem.* 73, 39–85
54. Liu, G., and Warbrick, E. (2006) *Biochem. Biophys. Res. Commun.* 349, 360–366
55. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) *EMBO J.* 19, 94–102
56. Painter, R. B. (1985) *Mutat. Res.* 145, 63–69
57. Norbury, C. J., and Zhivotovsky, B. (2004) *Oncogene* 23, 2797–2808
58. Latonen, L., and Laiho, M. (2005) *Biochim. Biophys. Acta* 1755, 71–89
59. Heffernan, T. P., Simpson, D. A., Frank, A. R., Heinloth, A. N., Paules, R. S., Cordeiro-Stone, M., and Kaufmann, W. K. (2002) *Mol. Cell. Biol.* 22, 8552–8561
60. Chastain, P. D., II, Heffernan, T. P., Nevis, K. R., Lin, L., Kaufmann, W. K., Kaufman, D. G., and Cordeiro-Stone, M. (2006) *Cell Cycle* 5, 2160–2167
61. Liu, P., Barkley, L. R., Day, T., Bi, X., Slater, D. M., Alexandrow, M. G., Nasheuer, H. P., and Vaziri, C. (2006) *J. Biol. Chem.* 281, 30631–30644
62. Luciani, M. G., Oehlmann, M., and Blow, J. J. (2004) *J. Cell Sci.* 117, 6019–6030
63. Rodriguez-Bravo, V., Guaita-Esteruelas, S., Florensa, R., Bachs, O., and Agell, N. (2006) *Cancer Res.* 66, 8672–8679
64. Helt, C. E., Cliby, W. A., Keng, P. C., Bambara, R. A., and O’Reilly, M. A. (2005) *J. Biol. Chem.* 280, 1186–1192