Mon- and Polyubiquitination*

Differential Regulation of Rad18 through Rad6-dependent Mono- and Polyubiquitination*

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Rad18 is involved in postreplication repair mainly through monoubiquitination of proliferating cell nuclear antigen (PCNA). Here we show that Rad18 protein was detected in human cells as two major bands at 75 and 85 kDa by Western blot. The bands were identified as nonubiquitinated and monoubiquitinated forms of Rad18, respectively, by mass spectrometry. Multiple ubiquitinated bands of Rad18 were detected in vitro in the presence of E1, E2 (Rad6), and methylated ubiquitin, indicating that Rad18 was monoubiquitinated at multiple sites through autoubiquitination. Rad18 self-associates, and this interaction was abolished by replacing one of the conserved cysteine residues with phenylalanine in the zinc finger domain (C207F). In the C207F mutant Rad18, monoubiquitination of Rad18 was not observed in vivo, suggesting that self-association was critical for monoubiquitination. Monoubiquitinated Rad18 was detected mainly in the cytoplasm, whereas nonubiquitinated Rad18 was detected predominantly in the nuclei. Furthermore, Rad18 was shown to be polyubiquitinated in cells treated with proteasome inhibitors. Purified Rad18 was also polyubiquitinated in an in vitro system containing E1, E2 (Rad6), and ubiquitin, and it was degraded by the addition of proteasomes. These results suggest that the amount of Rad18 in the nucleus is regulated differentially by mono- and polyubiquitination.
100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37 °C in a 5% CO2 humidified atmosphere.

**Plasmids**—Human RAD18 cDNA was amplified with a cDNA expression library derived from a human epithelial cell line. The 5′-primer contained an EcoRI site as well as a sequence encoding for a Myc epitope, and the 3′-primer contained EcoRI site. T7-tagged (MASMTGG-GqQMG) Rad6 and FLAG-tagged (MDYKDDDDKGS) ubiquitin expression constructs were generated similarly. A RAD18 construct tagged with three hemagglutinin (3XHA) epitopes was generated using two rounds of PCR. A fragment of RAD18 cDNA containing an HA epitope tag was amplified in the first round of PCR. In the second round of PCR, the 3XHA-tagged RAD18 construct was generated with a forward primer containing a 3XHA epitope tag sequence and using the first round PCR product as a template. The mammalian expression plasmid (pCAGGS) encoding wild-type RAD18, C28F Rad18, C207F Rad18, and Rad18 lacking the Rad6 binding domain have been described previously (6). A mammalian expression vector encoding wild-type RAD18, C28F RAD18, C207F RAD18, and RAD18 lacking the Rad6 binding domain were amplified using a RAD18-pCAGGS construct using a primer containing an EcoRI site and a sequence encoding the Myc epitope. The PCR product was cloned into pCAGGS (20). For isolation of stable transformants of RAD18−/− cells, Myc epitope-tagged wild-type RAD18, C28F RAD18, and C207F RAD18 were cloned into pcDNA3.1 (Invitrogen). Correct construction of the plasmids was confirmed by DNA sequencing. For purification of FLAG fusion ubiquitin, cDNA of ubiquitin was cloned into pCAL-n-FLAG (Stratagene).

**DNA Transfection—DNA transfection was carried out by using either electroporation (for COS-7 and GM637) or the FuGENE 6 transfection reagent (Roche Applied Science) for COS-7, GM637, and mouse RAD18−/− cells) according to the manufacturer’s instructions.

**Protein Purification—**Calmodulin-binding protein FLAG fusion ubiquitin was expressed in Escherichia coli (BL21-Gold(DE3)) and purified in columns according to standard instructions (Stratagene). GST-Rad18 was expressed in baculovirus-infected insect cells (Sf9), and purified using a glutathione column.

**Western Blot and Immunoprecipitation—**48 h after transfection of COS-7 or GM637 cells with the Rad18, Rad8, and, or control pCAGGS expression vectors, cell lysates were prepared in a buffer (0.25 M Tris-HCl, pH 6.8, 5% SDS, 50 mM glycerol, 0.01% bromphenol blue, and 0.5 M NaCl). After centrifugation, the supernatant was preincubated for 30 min at 4 °C and then centrifuged. The supernatants were mixed with an anti-HA antibody (G-Sepharose beads and clarified by centrifugation (15,000 rpm) at 4 °C. The beads were washed with 0.5 M LiCl in Tris-buffered saline followed by two additional washes with Tris-buffered saline. Proteins were loaded onto 7.5% SDS-PAGE followed by immunoblot analysis with anti-Rad18 antibody and ECL detection.

**In Vitro Ubiquitination Assays—**For a standard assay, immunoprecipitated 3XHA-Rad18 was mixed with three other components in various combinations in 30 μl of reaction buffer (25 mM Tris-HCl, pH 7.6, 1 mM MgCl2, 2 mM ATP, 0.5 mM dithiothreitol) and incubated for 60 min at 37 °C. The amounts of these components were 100 ng of E1 (Boston Biochem), 200 ng of GST-Rad6 (Boston Biochem), and 5 μg of either bacterially produced FLAG-ubiquitin or methylated ubiquitin (Calbiochem). For a 26 S proteasome degradation assay, the reaction mixture was incubated for another 30 min in the presence of 50 nm 26 S proteins (Boston Biochem). The reaction was stopped by the addition of SDS sample buffer and analyzed by Western blot with either an anti-Rad18 antibody or anti-HA antibody (HA-11) using a 7.5% acrylamide gel. For the ubiquitination assay with purified Rad18, reactions were performed as described above except for replacement of the HA-18 immunocomplexes with 30 ng of Rad18 purified from baculovirus-infected cells.

**Yeast Two-hybrid Assay—**Protein-protein interactions were examined using the yeast two-hybrid assay Matchmaker Gal4 II System (Clontech). Two plasmids, pACT2 (GAL4AD) and pGBK7 (GAL4BD) containing wild-type hRAD18 or mutated C207F hRAD18 cDNA, respectively, were introduced into the yeast strain Y187. All combinations were tested for β-galactosidase activity by the colony filter lift assay according to the manufacturer’s protocol.

**Nonidet P-40 Extraction—**To isolate the Nonidet P-40-insoluble fraction, GM637 cells were rinsed twice in cold PBS and incubated in PBS containing 0.1% Nonidet P-40 for 5 min on ice with gentle shaking. After rinsing with PBS, adhering cellular materials (Nonidet P-40-insoluble fraction) were lysed with an anti-HA antibody for 30 min. For double staining for Rad18 and Rad6, an antibody for Rad6 was used in this study. Rabbit polyclonal antibody against human Rad18 has been described elsewhere (6).

**Preparation of Cytoplasmic and Nuclear Fractions—**GM637 cells were harvested, rinsed with PBS, and pelleted. The cells were then suspended in 5 volumes of a cold hypotonic buffer A (20 mM Tris-HCl, pH 8.0, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride) and allowed to swell for 10 min on ice. After centrifugation at 200 × g for 10 min, the cells were resuspended in 2 volumes of buffer A with a tight glass homogenizer. The homogenate was spun at 200 × g for 10 min, and the supernatant (cytoplasmic fraction) was collected. Precipitated nuclei were suspended in 3 volumes of buffer A. Equal volumes of the cytoplasmic fraction and the nuclear suspension were solubilized individually with a sample application buffer. Total cell lysates were prepared in the same way. For analysis by Western blot, equal volumes of the cytoplasmic and nuclear Rad18 on the gel.

**Immunofluorescence Analysis—**Transfected cells were washed twice with PBS, pre-fixed with 3.7% paraformaldehyde in PBS for 5 min, washed again with PBS, and fixed with 80% methanol for 10 min at −20 °C. After blocking with PBS containing 0.05% bovine serum albumin and 0.05% Nonidet P-40, cells were stained with a specific primary antibody for 30 min. For double staining, cells were washed with PBS, and the secondary antibody (species-specific fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies for 30 min and then analyzed by fluorescence microscopy.

**Colonoy Forming Assay—**RAD18−/− cells were transfected with pcDNA3.1/Mygro encoding either wild-type Rad18 or C207F mutant by using FuGENE 6 and selected in medium containing 50 μg/ml hygromycin (Invitrogen). The surviving colonies were cloned. Expression of Rad18 was confirmed by Western blot using an anti-Rad18 antibody. These transformed RAD18−/− cells were cultured for 4 h to allow settlement and treated with different DNA-damaging agents. Cells were treated with MMC or MMC for 1 h, but cells treated using cisplatin were exposed continuously to cisplatin in the medium. Cells were incubated for 5 days, and then the surviving colonies were fixed with 3.7% formaldehyde, stained with 3% Giemsa solution, and counted. TriPLICATE dishes were used for each point, and the assay was repeated at least three times.
FIG. 1. Monoubiquitination of Rad18 in human cells. A, two Rad18 bands were identified in human cells. Rad18 protein in various human cells was detected by Western blot (IB) with an anti-Rad18 antibody (top panel). A low molecular mass isoform (75 kDa) and a high molecular mass isoform (85 kDa) were detected in all cells. Lane 1, primary fibroblasts; lane 2, A549; lane 3, MCF7; lane 4, Saos2; lane 5, HeLa; lane 6, XP2SASV; lane 7, GM637. Loading of equivalent cell lysates was confirmed by Western blot using an anti-α-tubulin antibody (bottom panel). B, identification of the high molecular mass isoform (85 kDa) as ubiquitinated Rad18. HA-tagged Rad18 was expressed in COS-7 cells, immunoprecipitated with anti-HA antibody, separated by SDS-PAGE, and visualized with Coomassie Blue. The high molecular mass isoform (85 kDa) of HA-Rad18 was excised, subjected to in-gel tryptic digestion, and analyzed by mass spectrometry. The resulting peptide mass spectrum is shown. Peaks indicated with numbers represent peptides derived from ubiquitin (peaks 1–4) and Rad18 (peaks 5 and 6). C, GM637 cells were transfected with a plasmid encoding FLAG-ubiquitin and incubated for 48 h. Rad18 was immunoprecipitated (IP) with either an anti-Rad18 or an anti-FLAG antibody and detected by immunoblotting with an anti-Rad18 antibody. Arrows indicate nonubiquitinated and monoubiquitinated Rad18.
RESULTS

Monoubiquitination of Rad18—To detect Rad18 protein in various human cell lines, we carried out Western blot analysis using an anti-Rad18 antibody. In all cells, Rad18 was detected as two major bands with molecular masses of about 75 and 85 kDa (Fig. 1A). However, the amounts and the ratios of these two bands varied from cell to cell: generally rapidly growing cells had more Rad18 protein than slowly growing ones, such as primary cells, and the ratios of 85 kDa to 75 kDa ranged from 1/10 to 1/2. Initially, we thought that the different sizes were caused either by phosphorylation or alternative splicing of the premature RAD18 mRNA. However, both possibilities were ruled out because dephosphorylation of the immunoprecipitated Rad18 proteins with phosphatase did not result in a monoubiquitination pattern either by phosphorylation or alternative splicing of the premature RAD18 mRNA. However, both possibilities were ruled out because dephosphorylation of the immunoprecipitated Rad18 proteins with phosphatase did not result in a monoubiquitination pattern (21, 22). When Rad18 was shown). To characterize the nature of the larger protein, HA-tagged Rad18 was overexpressed in COS-7 cells and immunoprecipitated with an anti-HA antibody. Precipitated proteins were separated by SDS-PAGE and stained with Coomassie Blue. The two major bands were excised and subjected to analysis by MALDI-TOF mass spectrometry and nano-LC/MS/MS (Fig. 1B). Both bands proved to be derived from Rad18 because digested peptides coincided with the expected sequences of Rad18. The upper band contained additional peptides corresponding to the ubiquitin sequences, whereas the lower band did not have these peptides (Table I). Given the difference of about 10 kDa in the molecular mass of the two proteins, we concluded that the upper band was derived from monoubiquitination of the lower band. From the mass spectrometry data, however, we could not determine the Rad18 lysine residue conjugated to ubiquitin. To confirm the ubiquitination of Rad18, a FLAG-tagged ubiquitin vector or an empty vector was transfected into human cells, and immunoprecipitation was performed with either an anti-Rad18 antibody or an anti-FLAG antibody. Proteins were separated by SDS-PAGE, and Rad18 was detected with an anti-Rad18 antibody. The upper band proved to be ubiquitinated Rad18 because only the upper band was immunoprecipitated with the anti-FLAG antibody (Fig. 1C, lane 1).

Rad6-dependent Monoubiquitination of Rad18—To determine whether the monoubiquitination of Rad18 is dependent on Rad6, Rad18 and various amounts of Rad6 expression vectors were transfected into COS-7 cells, and the effect of Rad6 on monoubiquitination was determined. Without Rad6 transfection, only a small fraction of Rad18 was monoubiquitinated (Fig. 2A, lane 1). However, the extent of monoubiquitination increased with increasing amounts of Rad6, suggesting that the reaction was dependent on Rad6 (Fig. 2A, lanes 2–4). To confirm that Rad6 functions as a ubiquitin-conjugating enzyme for the reaction, we employed an in vitro ubiquitination assay containing Rad18 purified from insect cells, Rad6, and ubiquitin. In this experiment, we used two forms of ubiquitin: methylated and nonmethylated. Methylated ubiquitin is known to block polyubiquitination (21, 22). When Rad18 was incubated with Rad6 and methylated ubiquitin, at least three bands of Rad18 were observed, indicating that Rad18 was monoubiquitinated at multiple sites and that Rad6 functions as a ubiquitin-conjugating enzyme (Fig. 2B, lanes 3 and 4). When ubiquitin was included in the reaction instead of methylated ubiquitin, smeared bands of Rad18 appeared above the monoubiquitinated bands (Fig. 2B, lane 5). Because specific ubiquitin ligase was not required for the reaction, we concluded that the monoubiquitination of Rad18 occurred through autoubiquitination.

Requirement of the Zinc Finger Domain for Monoubiquitination of Rad18—Rad18 is a multidomain protein bearing several structural motifs common to both lower eukaryotes and higher eukaryotes (14). In the NH₂-terminal half, Rad18 contains a C3HC4-type RING finger domain (amino acid residues 25–63) and a C2HC-type zinc finger domain (amino acid residues 204–223). In the middle portion, it has a Rad6 binding domain (amino acid residues 340–386). To determine the domain required for monoubiquitination of Rad18, we prepared plasmids bearing mutations in RAD18: C28F (replacement of the second cysteine in the RING finger domain with phenylalanine), C207F (replacement of the second cysteine in the zinc finger domain with phenylalanine), and DR6 (Rad18 lacking...
the binding domain for Rad6 (Fig. 3A), all of which contained a Myc tag on their NH2 termini. Myc-tagged wild-type Rad18 was expressed in RAD18−/− cells with increasing amounts of RAD6, and the amounts of monoubiquitinated Rad18 were determined (Fig. 3B). Without transfection of RAD6, only 10% of Rad18 was monoubiquitinated. The percentage of monoubiquitinated Rad18 increased to 40% with increasing amounts of exogenous Rad6, indicating that the reaction was dependent on Rad6. In contrast to wild type, the amount of monoubiquitinated C207F mutant Rad18 remained constant at very low levels with or without exogenous Rad6. The results raised two possibilities: (i) lysine residue (Lys218) in the zinc finger was the site of monoubiquitination and its monoubiquitination was disturbed by the mutation; (ii) the zinc finger domain was required for autoubiquitination. Because monoubiquitination of Rad18 was not affected by replacing the lysine residue in the zinc finger domain with arginine (K218R) (data not shown), the first possibility was unlikely. In the case of the C28F RING mutant, the total amount of protein was severely reduced, and mutants. The C28F RING mutant and C207F zinc mutant contain amino acid substitution at the 28th and 207th cysteine to phenylalanine, respectively. DR6 lacks the Rad6 binding domain (amino acids 340–386). WT, wild-type. B, Rad6-dependent monoubiquitination of Rad18. Wild-type (1.5 µg), C207F (1.5 µg), or C28F (1.5 µg) plasmid was cotransfected into RAD18−/− mouse fibroblasts with increasing amounts (0–1.5 µg) of T7-RAD6A plasmid. 24 h later, Rad18 and Rad6A proteins were detected by immunoblotting with an anti-Rad18 or an anti-T7-antibody, respectively. The same filter was blotted with an anti-α-tubulin antibody to confirm loading of equivalent cell lysates. C, defective monoubiquitination of the zinc mutant Rad18. Vector alone (lanes 1 and 5), wild-type RAD18 (lanes 2 and 6), C207F (lanes 3 and 7), or DR6 (lanes 4 and 8) plasmids were transfected into either COS-7 cells (left panel) or RAD18−/− mouse fibroblasts (right panel). Rad18 proteins were detected by immunoblot with an anti-Rad18 antibody. Arrowheads indicate monoubiquitinated Rad18. Note that endogenous monkey Rad18 in COS-7 cells was not detected under these blotting conditions because of weak cross-reactivity of the antibody against monkey Rad18 and relatively low abundance of endogenous Rad18 compared with overexpressed exogenous Rad18.

Function of the Zinc Finger Domain—in yeast, it has been reported that Rad18 self-associates through the zinc finger domain (23). To confirm that the zinc finger domain of mammalian Rad18 has the same function, a yeast two-hybrid assay was performed (Fig. 4A). When both binding domain and activation domain were linked to wild-type RAD18 cDNA, a positive signal was observed. In contrast, the positive signal disappeared when at least one of the Rad18 proteins contained a C207F zinc finger mutation. These results suggest that wild-type Rad18 proteins interact with each other through the zinc finger domain and that the C207F mutant Rad18 was unable to self-associate or associate with wild-type Rad18. However, it remained possible that the negative signal obtained with the C207F mutant Rad18 in the yeast two-hybrid assay was caused by loss of function because of misfolding. To rule out this possibility, we established mouse RAD18−/− cells that stably expressed either wild-type or C207F mutant human Rad18 and examined monoubiquitination of PCNA in these cells. It has been confirmed that PCNA is monoubiquitinated after DNA damage in a Rad18- and Rad6-dependent manner (17). The
cells were irradiated with various doses of UV light and incubated for 4 h. PCNA monoubiquitination was detected by Western blot with an anti-PCNA antibody. Although PCNA monoubiquitination was not detected in both parental RAD18/H11002/ cells (data not shown) and transformed cells expressing the C28F mutant Rad18, similar amounts of PCNA monoubiquitination were detected in cells expressing the C207F mutant Rad18 compared with the amount in cells expressing wild-type Rad18 (Fig. 4B). These results indicate that the C207F mutation does not affect the enzymatic activity responsible for monoubiquitination of PCNA. Furthermore, the sensitivity of RAD18−/− cells stably expressing C207F to various DNA-damaging agents including UV light, MMC (a DNA cross-linking reagent), MMS (a DNA methylating reagent), and cisplatin (a DNA cross-linking reagent) was restored to the levels of cells expressing wild-type Rad18 (Fig. 4C). These results indicate that the C207F mutation causes neither misfolding nor affects sensitivity to at least some types of DNA-damaging agents.

Intracellular Localization of Monoubiquitinated Rad18—Protein monoubiquitination is implicated in various processes of the cell functions independent of proteasome proteolysis (9). As a critical factor for postreplication repair, Rad18 is located mainly in the nuclei (6). To elucidate the relevance of monoubiquitination of Rad18 in intracellular localization, we first examined the distribution of monoubiquitinated and nonubiquitinated Rad18 biochemically. Plated GM637 cells were treated with a mild concentration of Nonidet P-40 for a short time on ice. By this treatment, most of the cytoplasmic proteins were washed out from the plated cells. Cellular components
remaining in the dishes were analyzed by Western blot with an anti-Rad18 antibody (Fig. 5A). Almost all of the monoubiquitinated Rad18 was extracted selectively from the treated cells, but nonubiquitinated Rad18 remained in the treated cells. Comparable levels of extraction of monoubiquitinated Rad18 were observed from cells, irrespective of UV irradiation. When Nonidet P-40-treated cells were stained for Rad18, Rad18 was found to be retained in the nuclei at high levels similar to those in nontreated cells. In contrast, most of the cytoplasmic fluorescence disappeared after the Nonidet P-40 treatment (Fig. 5B). To confirm localization of the monoubiquitinated form of Rad18 predominantly in the cytoplasm, proteins in GM637 cells were fractionated into the nuclear and cytoplasmic fractions by centrifugation of homogenized cells and analyzed by Western blot by using an anti-Rad18 antibody (Fig. 5C). Nonubiquitinated Rad18 was recovered predominantly in the nuclear fraction, whereas almost all of the monoubiquitinated Rad18 was recovered in the cytoplasmic fraction. A minor fraction of nonubiquitinated Rad18 was also detected in the cytoplasmic fraction probably because of destruction of nuclei during homogenization. These results indicate that Rad18 accumulates in the cytoplasm after monoubiquitination. We assumed that endogenous Rad18 in the nucleus might translocate to the cytoplasm if we could enhance the fraction of monoubiquitinated Rad18 in living cells. For this purpose, Rad6 was overexpressed in GM637 cells, and cellular localization of Rad18 was examined immunohistochemically (Fig. 5D). Rad6 localizes both in the nucleus and the cytoplasm (25, 26). In more than 60% of cells overexpressing Rad6, a large amount of endogenous Rad18 relocalized in the cytoplasm. Such relocalization of Rad18 was not observed in cells transfected with an empty vector (Fig. 5D).

Polyubiquitination of Rad18—When Rad18 in human cells was examined by Western blot using an anti-Rad18 antibody, only non- and monoubiquitinated Rad18 were detected (Fig. 1A). However, longer exposure revealed multiple bands above the monoubiquitinated Rad18 (data not shown). These results suggest that Rad18 is degraded rapidly by proteasomes after polyubiquitination. To confirm this, human cells were cultured for 12 h in the presence of the 26 S proteasome inhibitors, ALLN or MG132, and Rad18 was detected by Western blot using an anti-Rad18 antibody (Fig. 6A). A ladder pattern with molecular masses larger than the monoubiquitinated Rad18 was observed with these treatments. To clarify the requirement of Rad6 for the process, an in vitro Rad18 ubiquitination assay was carried out. For this experiment, we purified human

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**Fig. 5.** Cytoplasmic localization of monoubiquitinated Rad18. A, GM637 cells were irradiated with UV light (15 J/m²), incubated for the indicated times, treated with or without 0.1% Nonidet P-40 (NP-40) for 5 min, and washed with PBS. Cells remaining attached to the dishes were used for the assay. Rad18 was detected by immunoblotting (IB) with an anti-Rad18 antibody. Topoisomerase II (Topo II) and α-tubulin were included as a nuclear scaffold and a cytoplasmic marker, respectively. B, GM637 cells were treated with or without 0.1% Nonidet P-40. Adhering cells were fixed with paraformaldehyde and stained for Rad18 with an anti-Rad18 antibody. C, subcellular localization of monoubiquitinated Rad18. GM637 cells were disrupted by homogenization, and cytoplasmic and nuclear fractions were separated as described under “Experimental Procedures.” Rad18 in each fraction was detected by Western blot. Topoisomerase II and α-tubulin were included as a nuclear scaffold and a cytoplasmic marker, respectively. T, total cell lysate; C, cytoplasmic fraction; and N, nuclear fraction. D, cytoplasmic localization of Rad18 by overexpression of Rad6. A control vector (left panel) or plasmid encoding RAD6 cDNA (right panel) was transfected into GM637 cells. Localization of Rad18 and Rad6 was determined by double immunostaining by using an anti-Rad18 antibody (red) and an anti-UBC2 antibody (green). N, nuclear localization of Rad18, NC, nuclear and cytoplasmic localization of Rad18. The lower panel indicates the percentages of cells showing the staining pattern of N or NC for each transfection. For RAD6 transfection, only cells overexpressing Rad6 were counted. At least 200 cells were counted for each transfection.
Rad18 from insect cells using a recombinant baculovirus system. E1, E2 (Rad6), Rad18, and ubiquitin were included in the reaction mixture in various combinations, and Rad18 was detected by Western blot with an anti-Rad18 antibody. The precipitated HA-Rad18 was incubated with E1 protein, E2 protein (GST-Rad6), and ubiquitin in the indicated combinations (lanes 1–4). The reaction products were incubated further with a 26 S proteasomal extract for 30 min (lanes 5–8). Rad18 was detected by an anti-HA antibody. D, reduced polyubiquitination of C207F Rad18. Wild-type, C207F, or C28F Rad18 was transiently expressed in COS-7 cells and immunoprecipitated (IB) with an anti-HA antibody. The precipitated HA-Rad18 was incubated with E1 protein, E2 protein (GST-Rad6), and ubiquitin in the indicated combinations (lanes 2, 3, and 4). In contrast, when Rad18 was incubated in the presence of all components, smeared polyubiquitinated Rad18 bands appeared (Fig. 6B, lane 5). In the reaction without Rad6, a small amount of additional ubiquitinated Rad18 band was detected (Fig. 6B, lane 3). Probably, a small amount of endogenous insect Rad6 copurified with human Rad18 through protein-protein interactions (Fig. 6B, lanes 1 and 3) caused this ubiquitination. These results suggest that Rad18 was polyubiquitinated by autoubiquitination in a Rad6-dependent manner. To test whether polyubiquitinated Rad18 was degraded by proteasomes, HA-tagged Rad18 was recovered from transfected COS-7 cells by immunoprecipitation, and a polyubiquitination reaction was performed in the in vitro reaction system used in Fig. 6B. Basically, the results were similar to those obtained in Fig. 6B: smeared polyubiquitinated bands were observed only when four components (E1, E2, Rad18, and ubiquitin) were included in the reaction (Fig. 6C, lane 4). When a 26 S proteasomal extract was added to the reaction product and incubated for 30 min, the smeared polyubiquitinated bands disappeared, suggesting that polyubiquitinated Rad18 was degraded by proteasome. (Fig. 6C, lanes 4 and 8). A band detected above the regular monoubiquitinated Rad18 (Fig. 6C, lanes 2, 4, 6, and 8) seemed to be Rad18 monoubiquitinated by coinmonoprecipitated monkey Rad6 at multiple sites. This band was refractory from degradation by proteasome. Finally, we determined the effect of the Rad18 mutations on polyubiquitination. RAD18 finger domain was also required for polyubiquitination (Fig. 6D, lanes 1–3). Under the transient overexpression conditions, a small fraction of the C207F zinc mutant of Rad18 was found to be monoubiquitinated in the absence of the inhibitor (Fig. 6D, lane 4). However, the amounts of this band changed little during culture, and only faint amounts of polyubiquitinated bands were detected even 24 h after the treatment, suggesting that the zinc finger domain was also required for polyubiquitination (Fig.
Two Rad18 molecules interact through the zinc finger domain (zinc) and monoubiquitinate each other. Precise direction of the two Rad18 molecules remains to be resolved.

In sharp contrast, the C28F RING finger mutant Rad18 was stabilized dramatically with MG132. Amounts of nonubiquitinated, monoubiquitinated, and polyubiquitinated forms of Rad18 almost comparable with those of wild-type Rad18 accumulated 24h after the treatment (Fig. 6D, lanes 4–6). These results suggest that C28F mutant Rad18 is an unstable protein and subject to degradation through ubiquitination by the protein quality control system (27) rather than the Rad6-Rad18 pathway.

**DISCUSSION**

In this study, we found that Rad18 was monoubiquitinated in human cells. Rad18 monoubiquitination was also reconstituted in an *in vitro* system including E1, Rad6 (E2), Rad18, and methylated ubiquitin, indicating that this Rad18 monoubiquitination occurs by autoubiquitination. In yeast, it was proved that Rad18 self-associates through the zinc finger domain (23). Self-association between wild-type human Rad18 was detected in our yeast two-hybrid assay (Fig. 4A), but it was abolished when one of the cysteine residues in the zinc finger domain was replaced with phenylalanine (C207F). C207F Rad18 was not monoubiquitinated in either wild-type or *RAD18*−/− cells (Fig. 3C). These results suggest that Rad18 interaction through the zinc finger domain is critical for Rad18 monoubiquitination. Fig. 7 shows our current model for the mechanism of Rad18 monoubiquitination. Two Rad18 molecules interact through the zinc finger domain, and one Rad18 molecule is ubiquitinated by the other Rad18 molecule with Rad6. The Rad18 mutant (DR6) lacking the binding domain for Rad6 was not monoubiquitinated in *RAD18*−/− cells, whereas it was monoubiquitinated in COS-7 cells. Because the DR6 mutant Rad18 contains a functional zinc finger domain, it would probably form a heterodimer with the endogenous monkey Rad18 in COS-7 cells, thereby allowing monoubiquitination of Rad18. In this model, we assume that when the concentration of Rad18 becomes low in the nucleus, the chance of Rad18 self-association would be diminished, resulting in the dominance of the monoubiquitinated form. In contrast, when the concentration of Rad18 increases, Rad18 molecules would be monoubiquitinated efficiently through self-association.

We concluded that Rad18 monoubiquitination induces localization of Rad18 in the cytoplasm for three reasons. (i) Most of the monoubiquitinated Rad18 was extracted from cells by treatment with a mild concentration of Nonidet P-40 (Fig. 5A). (ii) Monoubiquitinated Rad18 was recovered in the cytoplasmic fraction after subcellular fractionation (Fig. 5B). (iii) Overexpression of Rad6 increased the amount of monoubiquitinated Rad18, and under these conditions Rad18 was induced to accumulate in the cytoplasm (Fig. 5C). There are at least two possibilities to explain the cytoplasmic localization of monoubiquitinated Rad18. (i) Rad18 translocates from the nucleus to the cytoplasm after monoubiquitination in the nucleus. (ii) Rad18 is shuttling between the nucleus and the cytoplasm, and upon monoubiquitination in the cytoplasm, its nuclear import is blocked. Further studies are required to distinguish these possibilities. We assume that monoubiquitination of Rad18 is not involved directly in postreplication repair but is involved in the regulation of the amount of Rad18 in the nucleus for the following reasons. (i) Stable transformants of *RAD18*−/− cells expressing only nonubiquitinated Rad18 (C207F mutant) exhibited normal PCNA monoubiquitination and restoration of sensitivity to various DNA-damaging agents. (ii) The protein levels of the 75- and 85-kDa Rad18 species remained constant after UV irradiation (Fig. 5A). (iii) After cell fractionation, monoubiquitinated Rad18 was found predominantly in the cytoplasm.

Under nonstressed conditions, Rad6 functions as an E2 through interaction with at least three RING finger proteins, Ubr1, Bre1, and Rad18. Ubr1/Rad6 is involved in N-end rule protein degradation (15), whereas Bre1/Rad6 is involved in monoubiquitination of histone 2B (16), leading to chromatin remodeling. Thus, Rad6 interacts with multiple RING proteins in normal processes. It may be reasonable to speculate that maintenance of a proper concentration of Rad18 in the nucleus is important for various processes controlled by Rad6 and that exclusion of monoubiquitinated Rad18 from the nucleus functions for maintenance. Alternatively, monoubiquitination of Rad18 may function as a reservoir of excess Rad18, keeping it out in the cytoplasm, and when a large amount of Rad18 is required, it may be deubiquitinated and recruited to the nuclei.

We observed at least three bands in an *in vitro* ubiquitination assay with methyl-blocked ubiquitin which blocks polyubiquitination (21, 22). These data suggest that human Rad18 contains at least three potential lysine residues for monoubiquitination, whereas one of them seems to be monoubiquitinated dominantly under regular conditions. To determine the lysine residues to be monoubiquitinated, we replaced some lysine residues with arginine either individually or in combination. These include lysines 17, 52, 57, 73, 83, 127, 169, 197, 201, 218, 344, 345, 359, 362, 363, 376, 407, 462, and 491. However, we still detected monoubiquitinated Rad18 band in all cases and thus could not determine a specific site (data not shown).

Recently, it has been reported that some DNA repair-related proteins are monoubiquitinated under nonstressed or stressed conditions. Mono- and Polyubiquitination of Rad18

![Fig. 7. A model showing automonoubiquitination of Rad18. Two Rad18 molecules interact through the zinc finger domain (zinc) and monoubiquitinate each other. Precise direction of the two Rad18 molecules remains to be resolved.](Image 108x595 to 256x738)
by adding a proteasome extract (Fig. 6C). Thus, both mono- and polyubiquitination regulate the amount of functional Rad18 in the nuclei by different mechanisms. Because such autopolyubiquitination of RING proteins has been reported with XIAP, E6AP, MDM2, and BRCA1/BARD1, it might be a general property common to RING proteins (28–31). In RAD18Δ/z cells expressing the C207F zinc mutant of Rad18, the amount of polyubiquitinated bands seemed to be small compared with that in cells expressing wild-type Rad18 in the presence of a proteasome inhibitor (Fig. 6D). When we compared the amount of the nonubiquitinated form between the C207F mutant and wild-type Rad18, the amount of C207F was more abundant than that in wild-type Rad18 (Fig. 3B, compare lanes 1 and 5). Because the C207F Rad18 was not monoubiquitinated, the nonubiquitinated form Rad18 might accumulate in the nuclei. These results suggest that either monoubiquitination or cytoplasmic localization of Rad18 is required for polyubiquitination.

One of the remaining questions is why budding yeast Rad18 is not monoubiquitinated. Like mammalian Rad18, yeast Rad18 forms a tight complex with Rad6 (32), and self-associates through the zinc finger domain (23). It is possible that some critical lysine residue(s) for monoubiquitination in mammalian Rad18 is missing in the yeast, and that yeast cells have a different regulation system for Rad18 than that for mammalian cells.

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