A novel hydrogen peroxide-induced phosphorylation and ubiquitination pathway leading to RNA polymerase II proteolysis

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Abbreviations: RNAPII, RNA polymerase II; CTD, C-terminal domain; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; CDK, cyclin-dependent kinase; P-TEFb, positive transcription elongation factor b; TFIIH, transcription factor IIH; VHL, von Hippel Lindau tumor suppressor; TCR, transcription-coupled repair; NER, nucleotide excision repair; BER, base excision repair; CS, Cockayne syndrome; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.
SUMMARY

DNA damage-induced ubiquitination of the RNA polymerase II largest subunit Rpb1 has been implicated in transcription-coupled repair for years. The studies so far, however, have been limited to the use of bulky, helix-distorting DNA damages caused by UV light and cisplatin, which are corrected by the nucleotide excision repair (NER) pathway. Non-bulky, non-helix-distorting damages are caused at high frequency by reactive oxygen species in cells and corrected by the base excision repair (BER) pathway. Contrary to a classic view, we recently found that the second type of DNA lesions also causes RNA polymerase II stalling in vitro. In this paper, we show that hydrogen peroxide (H2O2) causes significant ubiquitination and proteasomal degradation of Rpb1 by mechanisms that are distinct from those employed after UV irradiation. UV irradiation and H2O2 treatment cause characteristic changes in protein kinases phosphorylating its carboxy-terminal domain at Ser-2 and Ser-5. The H2O2-induced ubiquitination is likely dependent on unusual Ser-5 phosphorylation by ERK1/2. Moreover, the H2O2-induced ubiquitination occurs on transcriptionally engaged polymerases without a help of CSA, CSB, and pVHL proteins, which are all required for the UV-induced ubiquitination. These results suggest that stalled polymerases are recognized and ubiquitinated differentially depending on the types of DNA lesions. Our findings may have general implications in the basic mechanism of transcription-coupled NER and BER.

Keywords: RNA polymerase II, ubiquitination, transcription-coupled repair, hydrogen peroxide, base excision repair, CTD phosphorylation, ERK1/2
INTRODUCTION

RNA polymerase II (RNAPII) is a large protein complex involved not only in transcription but also in other biological processes in the nucleus, including mRNA processing and transcription-coupled repair (TCR) (1, 2). RNAPII consists of twelve subunits. The largest subunit Rpb1 contains a unique C-terminal domain (CTD) that consists of the heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser that is repeated 26 times in yeast and 52 times in mammals. RNAPII undergoes at least three types of post-translational modifications, i.e. phosphorylation, ubiquitination, and glycosylation (3-7). These modifications are likely to play important roles in its regulation.

Of the modifications listed above, phosphorylation of the CTD has been the most studied and appreciated. The CTD is a target for several protein kinases including the cyclin-dependent kinase (CDK) 7, CDK8, CDK9, extracellular signal-regulated kinase (ERK), DNA-dependent protein kinase, and c-Abl (3). Besides the tyrosine kinase c-Abl that targets Tyr-1 of the CTD (8), the known CTD kinases predominantly target either Ser-2 or Ser-5. Though there is some inconsistency in literature, it is generally thought that CDK7, a component of the transcription factor (TF) IIH, is a major Ser-5 kinase, whereas CDK9, a component of positive transcription elongation factor (P-TEF) b, is a major Ser-2 kinase within cells (3, 9-11). Phosphates on the CTD are removed by CTD-specific phosphatases including Fcp1 (12). CTD phosphorylation is dynamically regulated during transcription cycle and thought to play important roles in efficient transcription elongation and mRNA processing. For example, TFIIH-mediated Ser-5 phosphorylation in the promoter-proximal region promotes mRNA capping (13). P-TEFb-mediated Ser-2 phosphorylation reverses transcription repression imposed by the negative transcription elongation factors (14). Little is known about the roles, if any, of the CTD phosphorylation by other kinases.

Rpb1 is also ubiquitinated upon exposure of cells to UV light or cisplatin (4). This modification leads to its rapid proteasomal degradation. While direct evidence is lacking, it is
suggested that the modification is involved in TCR (1, 4-6). The rationale is as follows. DNA lesions within the coding region often block the progression of RNA polymerases. Stalled polymerases are intrinsically inhibitory to DNA repair because they prevent access of repair enzymes to damaged sites. These polymerases therefore need to be removed, possibly by proteolysis. The process called TCR enables cells to remove DNA lesions in the coding region considerably faster than those in the genome overall. Cockayne syndrome A (CSA) and B (CSB) proteins are involved in TCR and, in accordance with their predicted roles, required for UV-induced ubiquitination and degradation of Rpb1 (4). Recently, Kuznetsova et al. have provided evidence that the von Hippel Lindau tumor suppressor protein (pVHL)-associated complex is the E3 enzyme responsible for UV-induced ubiquitination of Rpb1 (15). According to another report (16), pVHL also targets Rpb7 subunit of RNAPII for ubiquitination. UV-induced ubiquitination of Rpb1 appears to depend on its phosphorylation on the CTD. Supporting this idea is the observation that inhibition of CTD phosphorylation by the protein kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) prevents the UV-induced ubiquitination (17).

We seek to understand more about the covalent modifications of RNAPII. As for ubiquitination, the causal relationships between DNA lesions and ubiquitination and between ubiquitination and DNA repair remain to be elucidated. There are various types of DNA lesions that are corrected by different mechanisms. Therefore we have tested several DNA-damaging agents to see whether any of them induce Rpb1 ubiquitination. This search was fueled in part by a recent discovery that TCR is not limited to the NER pathway, which corrects DNA lesions induced by UV light and cisplatin (18). Among the DNA-damaging agents we tested, hydrogen peroxide (H₂O₂) significantly induced Rpb1 ubiquitination. H₂O₂ causes oxidative DNA damages that are mainly corrected by the BER pathway. In this paper, we describe characterization of H₂O₂-induced ubiquitination of Rpb1 and discuss its potential role in TCR of oxidatively damaged DNA.
EXPERIMENTAL PROCEDURES

Cell culture

HeLa human cervical carcinoma cells were maintained in minimal essential medium (Nissui) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. 786-O human renal carcinoma cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. Simian virus 40-immortalized human fibroblasts WI38VA13 (WT), CS3BE-SV (CSA), and CS1AN-SV (CSB) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. These cells were cultured under 5% CO2 at 37°C. In some experiments, cells were irradiated with 254 nm UV light at 15 J/m² using UV Stratalinker 2400 (Stratagene) or treated with 10 mM H2O2 (Sigma) for 15 min, and then recovered for various time. Where indicated, 50 µM DRB, 30 µM U0126, 5 µM SB203580, 30 µM SP600125, 100 µM MG132, or 25 µg/ml cycloheximide were added to cells 30 min before UV or H2O2 treatment. Stock solutions of DRB (10 mM, Sigma) and cycloheximide (10 mg/ml, Sigma) were prepared using 100% ethanol, whereas those of U0126 (10 mM, Calbiochem), SB203580 (10 mM, Calbiochem), SP600125 (20 mM, Calbiochem), and MG132 (10 mM, Sigma) were prepared using dimethylsulfoxide. In Fig. 5, cell viability was quantified by a soluble tetrazolium/formazan assay. Cells were trypsinized, 100 µl aliquots (equal to 10³~10⁴ cells) were incubated with 10 µl of the live cell counting reagent SF (Nacalai) for 1 h on 96-well plates, and absorbance at 450 nm was read using Microplate reader model 450 (Bio-rad).

Immunological analysis

To detect Rpb1, N20 (Santa Cruz), H5 (Covance), and H14 (Covance) were used. N20 recognizes the most N-terminus and therefore reacts with both the IIo and IIa forms of Rpb1. H5 and H14 recognize the Ser-2 and the Ser-5 phosphorylated CTD, respectively (19). To detect ERK1/2, anti-p44/p42 (Cell Signaling) and E10 (Cell Signaling) were used. The former
recognizes total ERK1/2, whereas the latter recognizes an active, Thr-202 and Tyr-204 phosphorylated form. In addition, anti-Lamin A/C antibody (346, Santa Cruz), anti-α-tubulin antibody (B-7, Santa Cruz), and anti-C/EBPβ antibody (198, Santa Cruz) were used. For immunoblotting, cells were washed with phosphate buffered saline and lysed with high salt buffer [50 mM Tris (pH 8.0), 500 mM NaCl, 1.0% NP-40]. Whole cell extracts (10 µg) were resolved by 7% or 10% SDS-PAGE, and transferred to PVDF membrane (Millipore). Immunoreactive bands were visualized by ECL (Amersham). For immunofluorescence microscopy, WI38VA13 cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min. Coverslips were incubated with primary antibodies at a 1:1000 dilution in phosphate buffered saline containing 1% bovine serum albumin and then with Fluorescein-conjugated anti-mouse IgM (1:200, ICN). Coverslips were mounted on slide glasses using Vectashield with DAPI (Vector) and examined under a fluorescence microscope (BX51, Olympus).

**Analysis of ubiquitinated proteins**

HF3 cells are HeLa derivative cells that constitutively express His- and Flag-tagged Rpb3. As shown previously (20), active RNAPII complex is purified to near homogeneity from the cells by anti-Flag affinity chromatography alone or in combination with Ni affinity chromatography. In Fig. 1B, whole cell extracts were prepared from HeLa or HF3 cells that were either untreated or treated with H2O2. The extracts (200 µg) were incubated with anti-Flag M2 agarose (Sigma) in high salt buffer for 2 h. The matrices were washed five times with the same buffer, and bound proteins were eluted with Flag peptide as recommended by the manufacturer (Sigma). The eluate materials were immunoblotted with anti-ubiquitin antibody (clone FK2, MBL) that specifically recognizes multi-ubiquitin chains.

In Fig. 1C, affinity matrices that are comprised of a GST-fusion protein containing the ubiquitin-associated (UBA) domains of Rad23 conjugated to glutathione agarose (Ubiquitinated
Protein Enrichment Kit, Calbiochem) were used for the enrichment of ubiquitinated proteins. The affinity matrices were incubated with untreated or H₂O₂-treated HeLa cell extracts (200 µg) in high salt buffer for 2 h. Then, the matrices were washed five times with the same buffer, and bound proteins were eluted with SDS loading buffer.

**Fractionation of cellular proteins**

Fractionation of cellular proteins was performed as described (21) with slight modification. WI38VA13 cells were trypsinized, washed with phosphate buffered saline, lysed in modified cytoskeleton (MCSK) buffer [10 mM Pipes (pH 6.8), 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin] containing 100 mM NaCl, 0.5% Triton X-100, and 1 mM DTT at 4°C for 10 min, and centrifuged at 7,500 x g for 3 min (S1). Pelleted nuclei were resuspended in MCSK buffer containing 50 mM NaCl and 0.5% Triton X-100, incubated in the presence of 1 mg/ml DNase I at 30°C for 1 h, and centrifuged at 7,500 x g for 3 min (S2). The pellet was extracted with MCSK buffer containing 2 M NaCl at 4°C for 15 min and centrifuged at 20,000 x g for 15 min to separate supernatant (S3) and pellet (P).
RESULTS

H₂O₂-induced ubiquitination of Rpb1

We examined whether any modification of Rpb1 occurs in H₂O₂-treated cells. HeLa cells were irradiated with 254 nm UV light at 15 J/m² or treated with 10 mM H₂O₂ for 15 min and recovered for various times. Then, whole cell extracts were prepared and subjected to immunoblot analysis using anti-Rpb1 antibody H14. H14 recognizes the Ser-5 phosphorylated CTD and also has been used to detect ubiquitinated Rpb1 following UV irradiation (4, 17). As shown in Fig. 1A, H₂O₂ treatment resulted in the appearance of smeared H14-reactive bands with a significantly slower mobility than that of the normally phosphorylated IIo form (lane 11). Consistent with earlier reports (4, 17), UV irradiation also caused the appearance of smeared bands (lane 4), which are ubiquitinated Rpb1 (data not shown). Ubiquitination of Rpb1 occurred transiently with a peak at 1 h after UV irradiation. The kinetics for the H₂O₂-induced modification is similar to that for the UV-induced ubiquitination. In contrast, the band pattern of the H₂O₂-induced modification is quite different from that of the UV-induced ubiquitination. Even at higher doses of UV, greater mobility shift of Rpb1 was not observed (data not shown), suggesting that the modifications induced by UV and H₂O₂ are qualitatively different.

To examine whether the H₂O₂-induced modification is ubiquitination, RNAPII was immunoprecipitated from extracts of untreated and H₂O₂-treated cells, and immunoblotted with anti-ubiquitin antibody that recognizes multi-ubiquitin chains. To avoid inclusion of irrelevant ubiquitinated proteins in the samples, we used HF3 cells, HeLa derivative cells that constitutively express His- and Flag-tagged Rpb3 (20), and purified tagged RNAPII complex by anti-Flag affinity chromatography. As shown by silver staining (Fig. 1B, left), RNAPII subunits and some impurities were found in the HF3 eluate fractions. As shown by immunoblot analysis (Fig. 1B, right), ubiquitinated proteins were present only in the H₂O₂-treated HF3 samples. Because anti-ubiquitin antibody did not react with the negative control samples that contained the same
impurities, ubiquitination likely occurred to RNAPII subunits. Smeared bands of >250 kDa, which are similar in size to the H2O2-induced, H14-reactive bands, may correspond to ubiquitinated Rpb1 (Fig. 1B, right). Smeared bands of ~160 kDa may be due to ubiquitination of other RNAPII subunits. To obtain direct evidence for Rpb1 ubiquitination, ubiquitinated proteins were affinity-purified from untreated and H2O2-treated cell extracts using a GST-fusion protein containing the UBA domains of Rad23, as a ligand. Rad23 strongly binds to ubiquitin through the conserved UBA domains (22). As shown in Fig. 1C, the H2O2-induced, H14-reactive species were highly concentrated by the affinity matrices, whereas the unmodified IIo form was washed off. These results prove the specificity of the procedure and demonstrate that the novel species of Rpb1 induced by H2O2 are ubiquitinated.

Rapid proteasomal degradation of Rpb1 after H2O2 treatment

According to recent reports (17), UV-induced ubiquitination of Rpb1 leads to its rapid proteasomal degradation. We therefore examined whether H2O2-induced ubiquitination of Rpb1 also causes its degradation. Subsequent to H2O2 treatment, the protein level of Rpb1 was monitored in the presence of the protein synthesis inhibitor cycloheximide with or without the proteasomal inhibitor MG132. As shown by immunoblot analysis using antibody N20 that reacts with total Rpb1, no significant change was observed in the protein levels of the IIo form and the unphosphorylated IIa form during 4 h after cycloheximide addition (Fig. 1D, lanes 1-8). Over the same period, however, the protein levels of the Ser-5 phosphorylated IIo form and the ubiquitinated form markedly decreased in the absence of MG132 (lanes 1-4). The protein levels of these forms remained unchanged in the presence of MG132 (lanes 5-8). These results suggest that subsequent to H2O2 treatment, the Ser-5 phosphorylated form, but not the Ser-2 phosphorylated form or the unphosphorylated form, is targeted for rapid proteasomal degradation through its ubiquitination.
H$_2$O$_2$-induced ubiquitination of Rpb1 depends on its Ser-5 phosphorylation by ERK1/2

Next we analyzed a role for RNAPII phosphorylation in the H$_2$O$_2$-induced ubiquitination. Four protein kinase inhibitors DRB (P-TEFb inhibitor), U0126 (MAPK/ERK kinase (MEK) 1/2 inhibitor), SB203580 (MAPK p38 inhibitor), and SP600125 (JNK inhibitor) were added to HeLa cells 30 min prior to UV irradiation or H$_2$O$_2$ treatment and left in culture during a 1-h recovery incubation (Fig. 2A, upper panels). Total ERK serves as a loading control. Under normal growth conditions, Ser-2 phosphorylation, detected by the monoclonal antibody H5, was strongly inhibited by DRB, whereas Ser-5 phosphorylation was affected by none of the inhibitors tested (lanes 1-5). These results are consistent with earlier observations that P-TEFb and TFIIH are major Ser-2 and Ser-5 kinases, respectively (3, 9-11). TFIIH kinase is not sensitive to DRB at a concentration used here.

UV irradiation and H$_2$O$_2$ treatment remarkably change protein kinases phosphorylating Ser-2 and Ser-5 without significantly affecting the overall phosphorylation level of Rpb1. After UV irradiation, Ser-2 and Ser-5 phosphorylation was sensitive to both DRB and SP600125 (Fig. 2A, lanes 6-10). After H$_2$O$_2$ treatment, Ser-2 phosphorylation was relatively insensitive to any of the inhibitors tested, whereas Ser-5 phosphorylation was highly sensitive to U0126 (lanes 11-15). Thus, the following conclusions are drawn (Fig. 2C). First, UV irradiation somehow broadens the substrate specificity of P-TEFb, allowing it to phosphorylate not only Ser-2 but Ser-5 of the CTD. Second, UV irradiation reveals a previously undescribed role of JNK as a CTD kinase. Third, H$_2$O$_2$ treatment causes a situation where Ser-5 phosphorylation is highly dependent on the MEK1/2 pathway. Given the several reports showing that ERK1/2, related MAPKs activated by MEK1/2, act as CTD kinases (9, 23, 24), we tentatively conclude that ERK1/2 are major Ser-5 kinases after H$_2$O$_2$ treatment.

We looked at activation of the MEK1/2-ERK1/2 pathway after UV irradiation or H$_2$O$_2$ treatment using an antibody against phospho-ERK1/2. ERK1/2 were transiently activated
immediately after either stimulation, which precedes RNAPII ubiquitination (Fig. 1A). As anticipated, ERK1/2 activation was suppressed by U0126, but not by the other kinase inhibitors tested (Fig. 2A, lower panels). Thus, ERK1/2 act as major Ser-5 kinases only after H$_2$O$_2$ treatment, although they are activated by both UV and H$_2$O$_2$.

The appearance of ubiquitinated Rpb1 is correlated well with the appearance of Ser-5 phosphorylated Rpb1. The UV-induced ubiquitination was suppressed by DRB and SP600125 (Fig. 2A, lanes 7 and 10), whereas the H$_2$O$_2$-induced ubiquitination was suppressed by U0126 (Fig. 2A, lane 13 and Fig. 2B). These results indicate that Ser-5 phosphorylation by ERK1/2 is important for H$_2$O$_2$-induced ubiquitination of Rpb1.

Distinct factor requirements for UV-induced and H$_2$O$_2$-induced ubiquitination of Rpb1

The results so far have established that UV-induced and H$_2$O$_2$-induced ubiquitinations of Rpb1 are mechanistically different. To extend this point further, we examined whether the H$_2$O$_2$-induced ubiquitination occurs in CSA, CSB, and VHL deficient cells. According to a previous report, CSA, CSB, and some other DNA repair factors are required for UV-induced ubiquitination of Rpb1 (4). In addition, a recent paper has described that pVHL, a component of the E3 ubiquitin ligase complex is responsible for the UV-induced ubiquitination (15). As shown in Fig. 3A, CSA and CSB deficient cells supported H$_2$O$_2$-induced Rpb1 ubiquitination as wild-type cells, whereas the mutant cells were unable to induce Rpb1 ubiquitination in response to UV irradiation. Similarly, the H$_2$O$_2$-induced ubiquitination occurred in VHL deficient cells as in wild-type cells (Fig. 3B). Therefore, CSA, CSB, and pVHL are all dispensable for H$_2$O$_2$-induced ubiquitination of Rpb1.

H$_2$O$_2$-induced ubiquitination occurs on transcriptionally engaged RNAPII

We next investigated whether H$_2$O$_2$-induced phosphorylation and ubiquitination occur on
transcriptionally engaged RNAPII. Transcriptionally engaged RNAPII is predominantly in the IIo form and usually resistant to various extraction procedures (21). Conversely, much of the non-extractable IIo form is considered to be transcriptionally engaged.

First, we compared subnuclear localization of the Ser-2 and the Ser-5 phosphorylated IIo forms of RNAPII before and after H$_2$O$_2$ treatment by immunofluorescence microscopy. The antibodies H5 and H14 both stain fine dots in the non-nucleolar portion of the nucleus, which significantly overlap with the sites of active transcription (25). As shown in Fig. 4A, the distribution of RNAPII did not change substantially after H$_2$O$_2$ treatment. Consistent with the above results, however, the H14 staining was sensitive to prior addition of U0126 (compare g and h), suggesting that ERK1/2 are responsible for Ser-5 phosphorylation after the H$_2$O$_2$ treatment.

Second, the extractability of RNAPII was compared before and after H$_2$O$_2$ treatment by biochemical fractionation (Fig. 4B). Cells were initially lysed with 0.5% Triton X-100 to release cytoplasmic proteins and soluble nuclear proteins. After centrifugation, the supernatant (S1) was found to contain cytoplasmic $\alpha$-tubulin, much of the transcriptional regulator C/EBP$\beta$, and ~30% of RNAPII, which is probably not engaged in transcription. Then, the pellet was treated with DNase I to solubilize chromatin proteins. After centrifugation, the supernatant (S2) contained the rest of C/EBP$\beta$ but no RNAPII. RNAPII was partially released at the next NaCl extraction step (S3), but a significant fraction of RNAPII remained in pellet (P) where most of Lamin A/C, structural components of the nuclear matrix, were found. For H$_2$O$_2$-treated cells, ubiquitinated Rpb1 was found in the S3 and P fractions (lanes 7 and 8). Moreover, the IIo form in these fractions was sensitive to U0126 (data not shown). These results indicate that H$_2$O$_2$-induced, ERK1/2-dependent phosphorylation and ubiquitination of Rpb1 occur on transcriptionally engaged RNAPII.

**U0126 selectively enhances H$_2$O$_2$-induced cell death**
If H$_2$O$_2$-induced phosphorylation and ubiquitination of Rpb1 are physiologically relevant, blocking this pathway should affect cell survival post H$_2$O$_2$ treatment, and it did (Fig. 5). U0126 or dimethylsulfoxide carrier was added to HeLa cells 30 min prior to UV irradiation or H$_2$O$_2$ treatment and left in culture until the cells were harvested 24 h post stimulation. Cell viability was quantified by cell counting (data not shown) and by a soluble tetrazolium/formazan assay (Fig. 5), which gave identical results. Under our conditions, U0126 alone showed no toxic effect on HeLa cell growth. H$_2$O$_2$ treatment caused cell death to some extent, and the presence of U0126 remarkably enhanced H$_2$O$_2$-induced cell death (Fig. 5A). UV irradiation also caused cell death in a dose-dependent manner, but U0126 did not enhance UV-induced cell death over the wide range of UV doses (Fig. 5A and B). The same was true when U0126 was removed 2 h post stimulation (data not shown), when the stress-induced phosphorylation and ubiquitination of Rpb1 would have completed without U0126 (Fig. 1A). Thus, the MEK1/2-ERK1/2 pathway plays an important role within the short time window when early cellular responses to oxidative stress including TCR occur. These results do not prove but support the idea that H$_2$O$_2$-induced phosphorylation and ubiquitination of Rpb1 are critical for cell survival.
DISCUSSION
UV and H2O2 are DNA-damaging agents causing different types of DNA lesions. Here we showed that, when cells are exposed to H2O2, RNAPII undergoes rapid ubiquitination and proteasomal degradation. Both UV and H2O2 cause transient ubiquitination of Rpb1 with a peak at 1 h post stimulation (Fig. 1). Despite the apparent similarity, UV- and H2O2-induced ubiquitinations of Rpb1 proceed via distinct pathways. First, the H2O2-induced ubiquitination involves ERK1/2, whereas the UV-induced ubiquitination involves P-TEFb and JNK (Fig. 2). Second, the H2O2-induced ubiquitination does not involve CSA, CSB, and pVHL, which are all required for the UV-induced ubiquitination (Fig. 3). Third, the band pattern of the H2O2-induced ubiquitination is quite different from that of the UV-induced ubiquitination (Fig. 1).

Dynamic change of protein kinases responsible for CTD phosphorylation
We showed that protein kinases phosphorylating the CTD change remarkably upon UV irradiation or H2O2 treatment (Fig. 2). The ‘replacement’ of the responsible kinases should occur through activation of unusual CTD kinases accompanied by inactivation of normal CTD kinases or activation of CTD phosphatases.

Surprisingly, P-TEFb is responsible for phosphorylation of not only Ser-2 but Ser-5 following UV irradiation (Fig. 2). This probably relates to the previous finding that P-TEFb is activated upon UV irradiation (26). Under uninduced conditions, a large proportion of P-TEFb in cells forms a catalytically inactive complex with 7SK RNA and MAQ1/HEXIM1 protein (27). This complex is dissociated upon several stimuli including UV irradiation, increasing the intracellular level of P-TEFb kinase activity. By contrast, ERK1/2 seem to be responsible for Ser-5 phosphorylation following H2O2 treatment (Fig. 2). The MAPKs ERK1/2 normally exist in an unphosphorylated, inactive form in the cytoplasm (28). Upon several stimuli including H2O2, ERK1/2 are phosphorylated at Thr-202 and Tyr-204 by the MAPKKs MEK1/2 and
translocated into the nucleus, where they phosphorylate the CTD. Nuclear localization of ERK1/2, however, does not suffice for CTD phosphorylation as illustrated by the fact that ERK1/2 do not work as CTD kinases after UV irradiation when ERK1/2 are activated and translocated into the nucleus equally well (28; see also Fig. 1).

The normal CTD kinase TFIIH is believed to phosphorylate Ser-5 concomitantly with transcription initiation. UV irradiation and H$_2$O$_2$ treatment probably inhibit both initiation and elongation of transcription and thereby prevent the initiation-associated Ser-5 phosphorylation by TFIIH. According to previous reports (13, 29), the phosphorylation status of the CTD is dynamically regulated during transcription elongation. Perhaps, RNAPII stalled at a DNA lesion is susceptible to phosphorylation and dephosphorylation on the CTD, and its phosphorylation status is determined by the opposing actions of the unusual CTD kinases described above and Fcp1 phosphatase.

**A possible role of H$_2$O$_2$-induced Rpb1 ubiquitination in transcription-coupled BER**

We propose a model that H$_2$O$_2$-induced Rpb1 ubiquitination is involved in transcription-coupled BER. Once thought of as a subpathway of NER, it is now clear that TCR also exists in BER (18, 30). Reactive oxygen species such as H$_2$O$_2$ induce non-bulky, non-helix-distorting DNA lesions such as 8-oxoguanine and thymine glycol that are mainly corrected by the BER pathway, whereas UV induces bulky, helix-distorting DNA lesions such as cyclobutane pyrimidine dimer and (6-4) photoproduct that are corrected by the NER pathway. As expected from the presence of transcription-coupled BER, oxidative DNA damages in the coding region are repaired considerably faster than those in the genome overall. Contrary to a classic view, we and others have shown recently that some of the H$_2$O$_2$-induced DNA lesions cause RNAPII stalling *in vivo* and *in vitro* (18, 31, 32), similar to the UV-induced DNA lesions. The data presented here suggest that such stalled polymerases are recognized and ubiquitinated.
differentially depending on the types of DNA lesions. It is reported that transcriptional arrest caused by the transcriptional inhibitor α-amanitin or by nucleotide starvation, free of any DNA damage, leads to Rpb1 ubiquitination \textit{in vitro} (6, 33). Thus, \textit{in vivo}, UV- or H$_2$O$_2$-induced arrest of elongating RNAPII itself may be the direct cause of Rpb1 ubiquitination. This view may be too simplistic, however, considering the qualitative difference of the UV- and H$_2$O$_2$-induced ubiquitination.

Ubiquitination of Rpb1 may enhance the TCR process by causing its proteasomal degradation and permitting access of the repair machinery to damaged sites. The differential ubiquitination may even serve as a switch between BER and NER by selectively recruiting repair factors of either pathway to damaged sites. Consistent with these scenarios, H$_2$O$_2$-induced ubiquitination and degradation of Rpb1 occur on transcriptionally engaged RNAPII (Fig. 4) concomitant with the progress of TCR (30), and inhibition of this process confers a H$_2$O$_2$-sensitive phenotype (Fig. 5) indicative of a TCR defect (34).

Not much is known about factors and mechanisms involved in transcription coupled BER. It is likely, however, that some protein factors including XPG and CSB, previously thought to be involved in NER, participate in transcription coupled BER (18). Interestingly, roles played by these common factors seem different between the pathways. XPG, an endonuclease cutting on the 3’ side of a DNA lesion during NER, functions independently of this activity during BER (35). We showed here that CSA and CSB are essential for UV-induced ubiquitination but not for H$_2$O$_2$-induced ubiquitination of Rpb1. The differential requirement for CSA and CSB may indicate their different roles in NER and BER.
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REFERENCES

1. Svejstrup, J. Q. (2002) *Nat. Rev. Mol. Cell. Biol.* **3**, 21-29

2. Shilatifard, A., Conaway, R. C., and Conaway, J. W. (2003) *Annu. Rev. Biochem.* **72**, 693-715

3. Oelgeschlager, T. (2002) *J. Cell. Physiol.* **190**, 160-169.

4. Bregman, D. B., Halaban, R., van Gool, A. J., Henning, K. A., Friedberg, E. C., and Warren, S. L. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 11586-11590

5. Mitsui, A. and Sharp, P. A. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 6054-6059

6. Lee, K. B., Wang, D., Lippard, S. J., and Sharp, P. A. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 4239-4244

7. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1993) *J. Biol. Chem.* **268**, 10416-10424

8. Baskaran, R., Dahmus, M. E., and Wang, J. Y. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11167-11171

9. Trigon, S., Serizawa, H., Conaway, J. W., Conaway, R. C., Jackson, S. P., and Morange, M. (1998) *J. Biol. Chem.* **273**, 6769-6775

10. Ramanathan, Y., Rajpara, S. M., Reza, S. M., Lees, E., Shuman, S., Mathews, M. B., and Pe’ery, T. (2001) *J. Biol. Chem.* **276**, 10913-10920

11. Prelich, G. (2002) *Eukaryot. Cell* **1**, 153-162

12. Yeo, M., Lin, P. S., Dahmus, M. E., and Gill, G. N. (2003) *J. Biol. Chem.* **278**, 26078-26085

13. Komarnitsky, P., Cho, E. J., and Buratowski, S. (2000) *Genes. Dev.* **14**, 2452-2460

14. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999) *Cell* **97**, 41-51

15. Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C., and Czyzyk-Krzeska, M. F. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 2706-2711
16. Na, X., Duan, H. O., Messing, E. M., Schoen, S. R., Ryan, C. K., di Sant’Agnese, P. A.,
Golemis, E. A., and Wu, G. (2003) *EMBO J.* **22**, 4249-4259
17. Luo, Z., Zheng, J., Lu, Y., and Bregman, D. B. (2001) *Mutat. Res.* **486**, 259-274
18. Le Page, F., Kwoh, E. E., Avrutskaya, A., Gentil, A., Leadon, S. A., Sarasin, A., and Cooper,
P. K. (2000) *Cell* **101**, 159-171
19. Patturajan, M., Schulte, R. J., Sefton, B. M., Berezney, R., Vincent, M., Bensaude, O.,
Warren, S. L., and Corden, J. L. (1998) *J. Biol. Chem.* **273**, 4689-4694
20. Hasegawa, J., Endou, M., Narita, T., Yamada, T., Yamaguchi, Y., Wada, T., and Handa, H.
(2003) *J. Biochem.* **133**, 133-138
21. Kamiuchi, S., Saijo, M., Citterio, E., de Jager, M., Hoeijmakers, J. H., and Tanaka, K. (2002)
*Proc. Natl. Acad. Sci. USA* **99**, 201-206
22. Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., Reed, S. I.
(2001) *Nat. Struct. Biol.* **8**, 417-422
23. Markowitz, R. B., Hermann, A. S., Taylor, D. F., He, L., Anthony-Cahill, S., Ahn, N. G., and
Dynan, W. S. (1995) *Biochem. Biophys. Res. Commun.* **207**, 1051-1057
24. Bonnet, F., Vigneron, M., Bensaude, O., and Dubois, M. F. (1999) *Nucleic Acids Res.* **27**, 4399-4404
25. Zeng, C., Kim, E., Warren, S. L., and Berget, S. M. (1997) *EMBO J.* **16**, 1401-1412
26. Nguyen, V. T., Kiss, T., Michels, A. A., and Bensaude, O. (2001) *Nature* **414**, 322-325
27. Michels, A. A., Nguyen, V. T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L., and
Bensaude, O. (2003) *Mol. Cell. Biol.* **23**, 4859-4869
28. Pouyssegur, J., Volmat, V., and Lenormand, P. (2002) *Biochem. Pharmacol.* **64**, 755-763
29. Cho, E. J., Kobor, M. S., Kim, M., Greenblatt, J., and Buratowski, S. (2001) *Genes. Dev.* **15**, 3319-3329
30. Cooper, P. K., Nouspikel, T., Clarkson, S. G., and Leadon, S. A. (1997) *Science* **275**, 990-
31. Tornaletti, S., Maeda, L. S., Lloyd, D. R., Reines, D., and Hanawalt, P. C. (2001) *J. Biol. Chem.* **276**, 45367-45371

32. Kuraoka, I., Endou, M., Yamaguchi, Y., Wada, T., Handa, H., and Tanaka, K. (2003) *J. Biol. Chem.* **278**, 7294-7299

33. Yang, L. Y., Jiang, H., and Rangel, K. M. (2003) *Int. J. Oncol.* **22**, 683-689

34. Unk, I., Haracska, L., Prakash, S., and Prakash, L. (2001) *Mol. Cell. Biol.* **21**, 1656-1661

35. Klungland, A., Hoss, M., Gunz, D., Constantinou, A., Clarkson, S. G., Doetsch, P. W., Bolton, P. H., Wood, R. D., and Lindahl, T. (1999) *Mol. Cell* **3**, 33-42
FIGURE LEGENDS

Fig. 1. H$_2$O$_2$-induced ubiquitination and degradation of Rpb1.

(A) HeLa cells were irradiated with 254 nm UV light at 15 J/m$^2$ (lanes 2-7) or treated with 10 mM H$_2$O$_2$ for 15 min (lanes 9-14) and recovered for indicated time. Then, whole cell extracts were prepared, and 10 µg aliquots were immunoblotted with indicated antibodies. Lanes 1 and 8 (C) show untreated cell extracts. Dots indicate the positions of stimulation-induced H14-reactive bands. An asterisk denotes a non-specific band observed occasionally. (B) Whole cell extracts were prepared from HeLa and HF3 cells that were either untreated or treated with H$_2$O$_2$. The extracts (200 µg) were incubated with anti-Flag M2 agarose, and after extensive washing, bound proteins were eluted with Flag peptide. Eluate materials were stained with silver or immunoblotted with anti-ubiquitin antibody. Asterisks denote non-specific bands irrelevant to RNAPII. (C) From untreated and H$_2$O$_2$-treated cell extracts (200 µg), ubiquitinated proteins were affinity-purified as described in Experimental Procedures. Purified materials (lanes 3 and 4) as well as one-tenth of the input materials (lanes 1 and 2) were immunoblotted with H14. Dots denote the positions of ubiquitinated Rpb1. (D) H$_2$O$_2$-treated cells were recovered for indicated time in the presence of 25 µg/ml cycloheximide. Where indicated (lanes 5-8), 100 µM MG132 was added 30 min prior to the H$_2$O$_2$ treatment and left in culture throughout the assay. Whole cell extracts (10 µg) were immunoblotted with indicated antibodies.

Fig. 2. H$_2$O$_2$-induced ubiquitination of Rpb1 depends on its Ser-5 phosphorylation by ERK1/2.

(A) DRB (50 µM), U0126 (30 µM), SB203580 (5 µM), SP600125 (30 µM), or carrier dimethylsulfoxide was added to HeLa cells 30 min prior to UV irradiation (15 J/m$^2$; lanes 6-10) or H$_2$O$_2$ treatment (10 mM for 15 min; lanes 11-15) and left in culture during a 1-h recovery incubation. Whole cell extracts (10 µg) were immunoblotted with indicated antibodies. Dots denote the positions of ubiquitinated Rpb1. (B) Experiments were performed as in (A) except that
different concentrations of U0126 were used. (C) Schematics showing which protein kinases are mainly responsible for CTD Ser-2 and Ser-5 phosphorylations in untreated (left), UV-irradiated (center), and H2O2-treated cells (right). Y, Tyr; S, Ser; P, Pro; T, Thr.

Fig. 3. Distinct factor requirements for UV-induced and H2O2-induced ubiquitination of Rpb1.
(A) WI38VA13 (WT), CS3BE-SV (CSA), and CS1AN-SV (CSB) cells were irradiated with UV light at 15 J/m2 or treated with 10 mM H2O2 for 15 min and recovered for 1 h. Whole cell extracts (10 µg) were immunoblotted with H14. Dots denote the positions of ubiquitinated Rpb1. (B) WI38VA13 (WT) and 786-O (VHL) cells were used for analysis as in (A).

Fig. 4. H2O2-induced ubiquitination occurs on transcriptionally engaged RNAPII.
(A) WI38VA13 cells grown on coverslips were treated or not with 10 mM H2O2 in the presence or absence of U0126 for 15 min and recovered for 1h. Cells were stained for RNAPII with H5 or H14 (green) and for DNA by DAPI (blue). Irrelevant non-nuclear immunostaining is seen occasionally. (B) WI38VA13 cells were treated or not with 10 mM H2O2 for 15 min, recovered for 1h, and subjected to biochemical fractionation as described in Experimental Procedures. Proteins of 0.5% Triton X-100 supernatant (S1), DNase I supernatant (S2), 2 M NaCl supernatant (S3), and pellet (P) fractions that were prepared from equivalent cell numbers were immunoblotted with indicated antibodies. Dots indicate the positions of ubiquitinated Rpb1.

Fig. 5. U0126 selectively enhances H2O2-induced cell death.
(A and B) U0126 or dimethylsulfoxide carrier was added to HeLa cells 30 min prior to UV irradiation (15 J/m2 unless stated otherwise) or H2O2 treatment (10 mM for 15 min) and left in culture until the cells were harvested 24 h post stimulation. Cell viability was quantified by a soluble tetrazolium/formazan assay. The y-axis shows a measured absorbance at 450 nm in
arbitrary unit. Open and filled boxes indicate the absence and presence of U0126.
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Fig. 2. Inukai et al.
Fig. 4. Inukai et al.
Fig. 5. Inukai et al.
A novel hydrogen peroxide-induced phosphorylation and ubiquitination pathway leading to RNA polymerase II proteolysis
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