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Original Contribution

THE RING-H2–FINGER PROTEIN APC11 AS A TARGET OF HYDROGEN PEROXIDE

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Abstract—The anaphase-promoting complex (APC) is a ubiquitin-protein ligase (E3) that targets cell cycle regulators such as cyclin B and securin for degradation. The APC11 subunit functions as the catalytic core of this complex and mediates the transfer of ubiquitin from a ubiquitin-conjugating enzyme (E2) to the substrate. APC11 contains a RING-H2–finger domain, which includes one histidine and seven cysteine residues that coordinate two Zn^{2+} ions. We now show that exposure of purified APC11 to H_2O_2 (0.1 to 1 mM) induced the release of bound zinc as a result of the oxidation of cysteine residues. It also impaired the physical interaction between APC11 and the E2 enzyme Ubc4 as well as inhibited the ubiquitination of cyclin B1 by APC11. The release of HeLa cells from metaphase arrest in the presence of exogenous H_2O_2 inhibited the ubiquitination of cyclin B1 as well as the degradation of cyclin B1 and securin that were apparent in the absence of H_2O_2. The presence of H_2O_2 also blocked the co-immunoprecipitation of Ubc4 with APC11 and delayed the exit of cells from mitosis. Inhibition of APC11 function by H_2O_2 thus likely contributes to the delay in cell cycle progression through mitosis that is characteristic of cells subjected to oxidative stress. Published by Elsevier Inc.

Keywords—Anaphase-promoting complex 11, Ubiquitin-protein ligase, RING finger, Cysteine oxidation, Zinc release, Hydrogen peroxide, Free radicals

INTRODUCTION

Ubiquitin-dependent proteolysis contributes to the precise control of cell cycle progression through mitosis by eliminating critical regulatory proteins [1–3]. The anaphase-promoting complex (APC), also known as the cyclosome, is an E3 ubiquitin-protein ligase that governs key events in mitosis, including the metaphase–anaphase transition and mitotic exit, by assembling multiubiquitin chains on mitotic regulators, thereby targeting them for destruction by the 26S proteasome. At the metaphase–anaphase transition, the APC initiates the separation of sister chromatids by catalyzing the ubiquitination of securin in the securin−separase complex [4]. The degradation of ubiquitinated securin then results in the liberation of separase, which cleaves a subunit of the cohesin complex and thereby triggers sister chromatid separation [5–7]. Exit of cells from mitosis requires the APC-mediated ubiquitination and destruction of B-type cyclins complexed with Cdc2, which results in inactivation of the kinase activity of Cdc2 [3,8,9].

Conjugation of ubiquitin to a substrate protein is a multistep process mediated by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (Ubc or E2), and a ubiquitin-protein ligase (E3) [10–12]. Ubiquitin is first activated by formation of a thiol–ester linkage with an E1 and is then transferred to an E2. The E3 transfers ubiquitin from the E2 to the substrate protein by promoting the formation of an isopeptide bond between the ubiquitin and a lysine of the target protein (or of another ubiquitin molecule that has already been

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linked to the target). The E3 thus interacts with both its cognate substrate and a specific E2.

On the basis of sequence homology and biochemical properties, E3 enzymes have been classified into those that contain a HECT (homologous to the E6-AP carboxyl terminus) domain and those that contain a RING (really interesting new gene) finger [13–15]. HECT-type E3s transfer ubiquitin to the target protein after forming an E3–ubiquitin thioester intermediate. In contrast, RING-finger–type E3s likely do not form such an intermediate but facilitate the transfer of ubiquitin directly from the E2 to the substrate. The RING-finger domains of E3s contain two zinc ions and serve as the catalytic core that forms part of the binding site for the E2 [16–18]. The RING-finger–type E3s are a diverse group of enzymes that include the APC [19–22], the Skp1–cullin1–F-box protein (SCF) complex [23–27], Siah [28,29], and RNF5 [30].

To date, the APC has been found to consist of 11 and 10 subunits in human and yeast, respectively [19,21]. APC11, which contains the zinc-coordinating domain referred to as the RING-H2 finger, is a catalytic core subunit of the APC [20–22]. Mutants of APC11 in yeast confer a mitotic arrest phenotype [19,20] similar to that associated with other APC mutants [19,31,32]. In the absence of any other subunit of the APC, APC11 alone is able to bind E2 (Ubc4) [20–22,33] and to support ubiquitination of cyclin B and securin [20–22].

All zinc fingers contain a core of highly conserved cysteine and histidine residues that tetrahedrally coordinate Zn^{2+}. Because the thiolate anion is more readily oxidized than is the protonated thiol, the zinc-bound cysteine thiolate anions ($Cys–S^-$) generally confer redox susceptibility to zinc-finger proteins, as is the case for heat shock protein 33 [34], protein kinase C [35,36], c-Raf kinase [37], the retinoic acid receptor [38], metallothionein [39], replication protein A [40], the transcription factor Sp-1 [41], and cellulose synthase [42].

We now show that hydrogen peroxide (H_2O_2) induces inactivation of APC11 through oxidation of the zinc-coordinating cysteines and the consequent release of zinc from the RING-H2 finger. This release of zinc impairs the ability of APC11 to bind Ubc4 and to transfer ubiquitin to APC substrates such as securin and cyclin B.

EXPERIMENTAL PROCEDURES

Materials

Mouse monoclonal antibodies to cyclin B1, to ubiquitin, to GST, or to the hexahistidine (His_6) epitope tag were from Santa Cruz Biotechnology, those to β-actin were from Abcam, and those to the Flag epitope tag were from Sigma. Rabbit polyclonal antibodies to securin were from Zymed, and those to APC11 were from Pharmingen. Rat antibodies to the hemagglutinin epitope (HA) tag and the pHM6 vector were from Roche Biochemicals. Bovine catalase was from Calbiochem. Propidium iodide and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate (AMS) were from Molecular Probes. Ubiquitin, *N*-ethylmaleimide (NEM), glucose oxidase, trichloroacetic acid (TCA), xylenol orange, ferrous ammonium sulfate, and perchloric acid were from Sigma. His_6-tagged ubiquitin, E1, and E2 (Ubc4) were from Boston Biochem, 4-(2-pyridylazo) resorcinol (PAR) was from Alfa Aesar, and Chelex-100 was from Bio-Rad. Glutathione–Sepharose, the pGEX-6P-1 vector, and PreScission protease were from Amersham Pharmacia Biotech, MG-132 was from Biomol, the pET15b and pET14b vectors were from Novagen, and the pCMV2B vector was from Stratagene. The pCGN vector, used for expression of proteins with an NH_2-terminal HA tag, was kindly provided by W. Herr. *p*-Hydroxymercuriphenylsulfonic acid (PMPS) was kindly provided by A. Ginsburg.

Expression plasmids

The DNA sequences for the human APC11 were amplified from HeLa cell cDNA and cloned into the *Bam*HI and *Eco*RI sites of pGEX-6P-1 for expression of GST-APC11. The DNA sequences for the NH_2-terminal 134 amino acids of human cyclin B1 and for human Ubc4 were amplified from HeLa cell cDNA and cloned into the *Nde*I and *Bam*HI sites of pET15b and the *Xho*I and *Bam*HI sites of pET14b, respectively, for expression of fusion proteins with an NH_2-terminal His_6 tag. For construction of the mammalian expression plasmids pCGN-APC11, pHM6-ubiquitin, and pCMV2B-Ubc4, the human APC11, ubiquitin, and Ubc4 cDNAs were amplified from HeLa cell cDNA and cloned into the *Xba*I–*Bam*HI sites of pCGN, the *Hind*III–*Eco*RI sites of pHM6, and the *Bam*HI–*Eco*RI sites of pCMV2B, respectively.

Expression and purification of recombinant proteins

Escherichia coli BL21 (DE3) carrying the plasmid encoding GST-APC11 was cultured at 37°C in LB medium supplemented with ampicillin (100 μg/ml) and $ZnCl_2$ (100 μM). Isopropyl-1-thio-β-D-galactopyranoside (0.2 mM) was added and the culture was incubated overnight at 20°C. GST-APC11 proteins were purified using glutathione–Sepharose (Amersham Pharmacia) and dialyzed against 10 mM HEPES–NaOH (pH 7.4). When indicated, GST was removed from the fusion protein by cleavage with PreScission protease. The concentration of the recombinant protein was determined by measurement of absorbance at 280 nm and based on an extinction coefficient of 2.105 for a solution of 1 mg/

ml [43]. The His_6-tagged cyclin B1(1–134) or Ubc4 proteins were also expressed in *E. coli* and were purified with the use of an immobilized nickel resin (Qiagen).

Zinc determination

Purified recombinant APC11 was dialyzed first against a solution containing 40 mM HEPES–KOH (pH 7.0), 2 mM DTT, and 100 μM $ZnCl_2$ and then against 40 mM HEPES–KOH (pH 7.0) alone. The release of zinc from the purified protein was measured as described [44] with an assay based on the formation of a complex between the released zinc and PAR. This complex absorbs light at 500 nm with an ε_{500} of 66,000 M^{-1} cm^{-1}. All solutions used for zinc determination were pretreated with Chelex-100 to remove background Zn^{2+}. The H_2O_2-induced release of zinc from APC11 was thus assayed with 0.1 mM PAR at 30°C in 200 μl of Chelex-100–treated 40 mM HEPES–KOH (pH 7.0). For determination of the total zinc content of untreated APC11, absorbance at 500 nm was monitored until a plateau was reached during the repeated addition of 5-μl portions of 0.5 mM PMPS to a mixture (1 ml) containing 1.5 μM APC11 and 100 μM PAR.

In vitro assay of ubiquitination and Ubc4 binding

GST-APC11 (4 μg) was incubated for 1 h at 30°C in the absence or presence of H_2O_2 in a final volume of 200 μl containing 50 mM HEPES–NaOH (pH 7.4). The reaction was stopped by the addition of 1 μg of catalase, and GST-APC11 was isolated with the use of glutathione–Sepharose. The beads were washed with 50 mM HEPES–NaOH (pH 7.4), and the immobilized fusion protein was assayed for its ability to ubiquitinate cyclin B1 or itself as well as its ability to bind Ubc4. The ubiquitination of cyclin B1 was measured in a 30-μl reaction mixture containing E1 (0.4 μg), Ubc4 (0.4 μg), ubiquitin (30 μg), 0.2 μg of His_6-tagged human cyclin B1(1–134), 2 mM ATP, 50 mM HEPES–NaOH (pH 7.4), 2.5 mM $MgCl_2$, and 1 mM DTT. For the autoubiquitination assay, the reaction mixture contained His_6-tagged ubiquitin (3 μg) instead of ubiquitin, and cyclin B1 was omitted. The reactions were performed for 1 h at 37°C and subjected to immunoblot analysis with antibodies to the His_6 tag. For the Ubc4 binding assay, the bead-immobilized GST-APC11 was incubated for 2 h at 4°C with His_6-Ubc4 (1 μg) in 1 ml of binding buffer consisting of 50 mM HEPES–NaOH (pH 7.0), 150 mM NaCl, 1% Nonidet P-40, and BSA (25 μg/ml). The beads were then washed four times with binding buffer and subjected to immunoblot analysis with antibodies to the His_6 tag.

Cell culture, transfection, and synchronization

HeLa cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). Transient transfection of cells was performed in 100-mm dishes with 12 μl of FuGENE 6 (Roche). After 36 h, the cells were arrested in M phase of the cell cycle by exposure to nocodazole as described previously [45]. They were washed and released in 60-mm dishes (6×10^5 cells per dish) containing serum-free medium.

Flow cytometry

Cells ($\sim 3 \times 10^5$) were washed twice with ice-cold PBS and then fixed overnight at 4°C in 70% ethanol. Immediately before flow cytometry, the cells were resuspended in PBS containing propidium iodide (50 μg/ml) and DNase-free RNase (10 μg/ml). They were then analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

Preparation of HeLa cell lysates

HeLa cells were washed twice with ice-cold PBS and then lysed for 10 min at 4°C in a solution containing 25 mM HEPES–NaOH (pH 7.4), 2 mM EGTA, 25 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, 5 mM NaF, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), 25 nM microcystin, and 50 μM MG-132. The lysate was centrifuged at 15,000 × *g* for 10 min, and the protein concentration of the resulting supernatant was measured by the Bradford assay.

Measurement of H_2O_2

The amount of H_2O_2 in medium was determined by the PCA-FOX assay [46]. Portions of medium (0.2 ml) were collected, mixed with 0.05 ml of 30% TCA, and then centrifuged at 15,000 × *g* for 10 min. The resulting supernatant (0.1 ml) was mixed with 0.9 ml of PCA-FOX solution (0.28 mM xylenol orange and 0.28 mM ferrous ammonium sulfate in 0.12 M perchloric acid) and incubated for 30 min at room temperature, after which the absorbance at 560 nm was measured and compared with a standard curve generated with dilutions of a standard H_2O_2 solution.

Separation of reduced and oxidized APC11 by a thiol-trapping procedure

After exposure to H_2O_2, cells were washed with ice-cold PBS, incubated on ice for 30 min with 10% TCA, scraped from the dish, and transferred to microcentrifuge tubes. After centrifugation at 15,000 × *g* for 5 min at 4°C, the pellets were washed once with 10% TCA and twice with acetone, solubilized in 0.1 ml of 200 mM Tris–HCl (pH 6.8) containing 1.5% SDS and 50 mM NEM, and incubated for 1 h at 30°C. The pH of the samples was adjusted to 8.5, and they were then incubated with 100 mM DTT for 30 min before dilution with 0.9 ml of 50 mM HEPES–NaOH (pH 7.2) containing

150 mM NaCl and 0.1 mM EDTA. Proteins were precipitated by the addition of 0.15 ml of 100% TCA and incubation on ice for 30 min. The samples were then centrifuged and washed as described above, and the pellets were finally solubilized in 100 mM Tris–HCl (pH 8.8) containing 1.5% SDS and 10 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate (AMS) and then incubated at 25°C for 2 h. The reaction was stopped by the addition of 5× nonreducing Laemmli buffer and the samples were subjected to immunoblot analysis.

RESULTS

H_2O_2 inhibits the ubiquitin ligase activity of APC11

In the absence of any other subunit of the APC, APC11 is able to mediate the ubiquitination both of itself and of APC substrates such as cyclin B and securin [20–22]. We first investigated whether H_2O_2 affects the autoubiquitination activity of APC11. After exposure to H_2O_2, recombinant GST-APC11 was incubated with His_6-tagged ubiquitin, E1, and Ubc4, and the formation of multiubiquitin chains was evaluated by immunoblot analysis with antibodies to the His_6 tag. Treatment with H_2O_2 resulted in a marked inhibition of the autoubiquitination activity of APC11 (Fig. 1A). We also examined the ubiquitin ligase activity of H_2O_2-treated GST-APC11 with a His_6-tagged NH_2-terminal fragment of human cyclin B1 that contains the destruction box required for proteolysis [47]. Again, H_2O_2 inhibited the ubiquitin ligase activity of APC11 in a concentration-dependent manner (Fig. 1B). Treatment of GST-APC11 with H_2O_2 also resulted in a marked inhibition of its ability to bind Ubc4 (Fig. 1C).

H_2O_2 induces zinc release from recombinant APC11

H_2O_2 induces the release of zinc ions from various zinc-finger–containing proteins, including heat shock protein 33 [34], protein kinase C [35,36], and c-Raf kinase [37]. We monitored Zn^{2+} release from APC11 with the use of the zinc-sensitive dye PAR. Incubation with 0.5 mM H_2O_2 indeed induced the release of Zn^{2+} from APC11; the release was slow, with a half-time of ~30 min (Fig. 2A). The maximal extent of Zn^{2+} release induced by H_2O_2 was determined to be 2.89 ± 0.17 mol per mole of APC11 (mean ± SE of values from three independent experiments). This value is consistent with the previous demonstration that APC11 contains a third Zn^{2+} binding site in addition to the two formed by the canonical RING-H2 finger [22]. The catalytic activity of APC11 was shown to be independent of the binding of zinc to this third site, however [20,22].

To demonstrate that the H_2O_2-induced release of zinc from APC11 was due to cysteine oxidation, we incubated APC11 with various concentrations of H_2O_2 and then subjected the recombinant protein to nonreducing SDS–

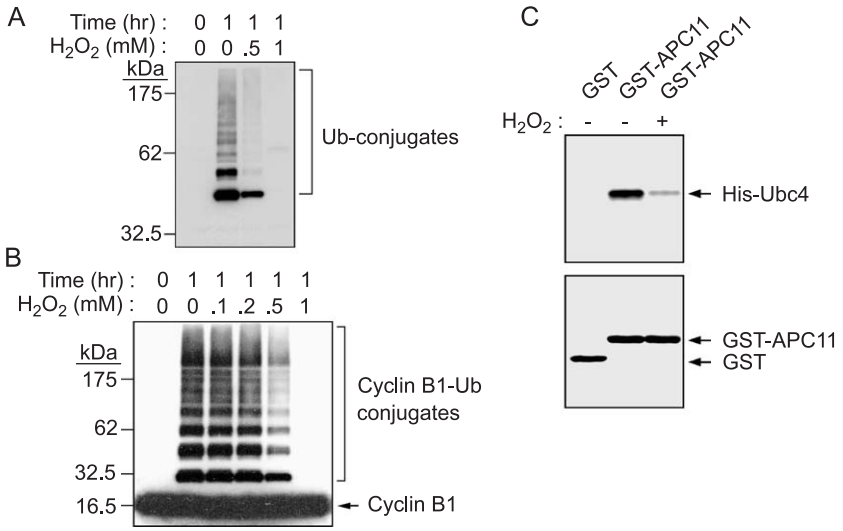

Fig. 1. Inhibition of the ubiquitin ligase activity of purified recombinant APC11 by H_2O_2. (A) Autoubiquitination assay. GST-APC11 was incubated for 1 h at 30°C with the indicated concentrations of H_2O_2, after which H_2O_2 was eliminated by the addition of catalase and the recombinant fusion protein was isolated by glutathione–Sepharose and incubated for the indicated times with purified E1, Ubc4, and His_6-ubiquitin in the presence of ATP. The reaction mixture was then subjected to immunoblot analysis with antibodies to the His_6 tag. The positions of protein–ubiquitin (Ub) conjugates are indicated on the right. (B) Ubiquitination of cyclin B1. GST-APC11 was treated with H_2O_2 as in (A) and then incubated with purified E1, Ubc4, ubiquitin, and His_6–cyclin B1(1–134) in the presence of ATP. The reaction mixture was analyzed as in (A). The positions of unmodified cyclic B1 and of cyclin B1–ubiquitin conjugates are indicated on the right. (C) GST or GST-APC11 was treated with H_2O_2 (500 μM) in (A), incubated with His-Ubc4 for 2 h at 4°C, and then isolated from reaction mixture by glutathione–Sepharose. The resulting precipitates were subjected to immunoblot analysis to His_6 tag (upper panel), after which the membrane was reprobed with antibodies to GST (lower panel). The positions of His-Ubc4 and GST-APC11 are indicated. Data in (A) through (C) are representative of three independent experiments.

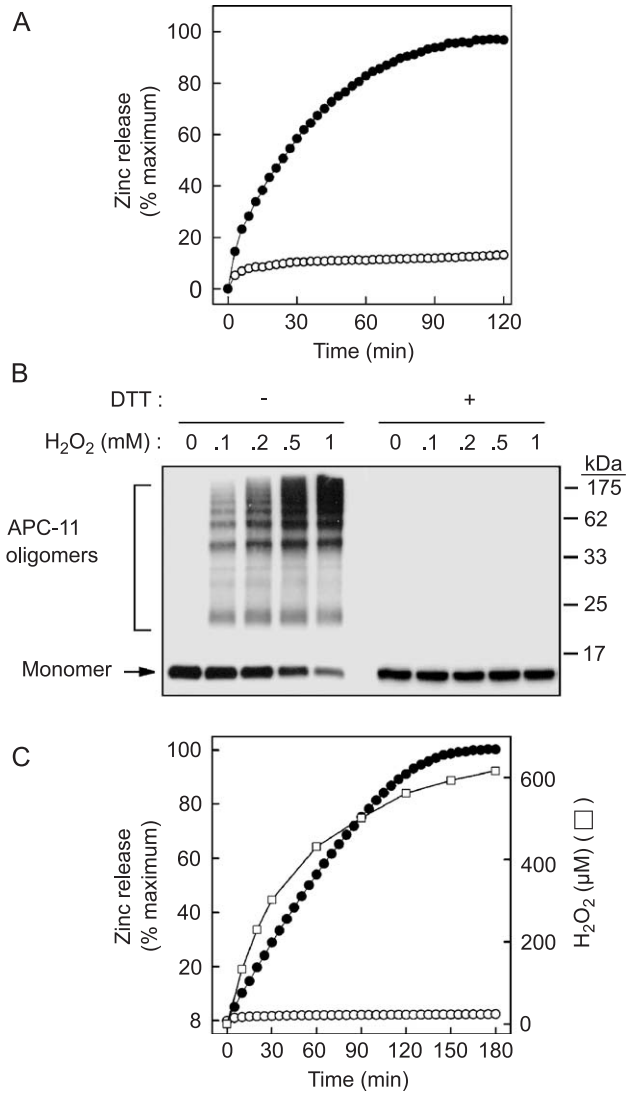

Fig. 2. The release of zinc from, and intersubunit disulfide formation by, recombinant APC11 induced by H_2O_2. (A) Purified recombinant APC11 (1 μM) was incubated for the indicated times at 30 °C with 40 mM HEPES–KOH (pH 7.0) in the presence of 0.1 mM PAR and in the absence (○) or presence (●) of 0.5 mM H_2O_2. The formation of the PAR_2–Zn^{2+} complex was monitored by measurement of absorbance at 500 nm. Zinc release was expressed as a percentage of the maximal value. (B) Purified recombinant APC11 (1 μM) was incubated at 30 °C first for 1 h with the indicated concentrations of H_2O_2 and then alkylated with 10 mM NEM. The reaction mixtures were incubated in the absence (−) or presence (+) of 50 mM DTT and then subjected to nonreducing SDS–PAGE followed by immunoblot analysis with antibodies to APC11. The positions of the APC11 monomer and oligomers are indicated on the left. Data in (A) and (B) are representative of three independent experiments. (C) Purified recombinant APC11 (1 μM) was incubated for the indicated times at 30 °C with 40 mM HEPES–KOH (pH 7.0) containing 150 mM glucose and 0.1 mM PAR and in the absence (○) or presence (●) of 40 mU of glucose oxidase. The reaction mixture was saturated with oxygen before the addition of glucose oxidase. Zinc release was measured as in (A) and expressed as a percentage of the maximal value. The concentration of H_2O_2 (□) in the reaction mixture was determined. Data are representative of three independent experiments.

PAGE with or without prior treatment with DTT. Treatment with H_2O_2 induced oligomerization of APC11 in a concentration-dependent manner, as revealed by electrophoresis of the sample not treated with DTT (Fig. 2B). Such oligomerization was not observed with the DTT-treated sample, indicating it was likely due to intersubunit disulfide formation.

H_2O_2 prevents destruction of APC substrates and delays cell cycle progression

We next tested whether H_2O_2 also inactivated APC11 in HeLa cells that had been synchronized in metaphase by treatment with nocodazole. The amounts of the APC substrates cyclin B1 and securin were greater in the metaphase-arrested cells than in asynchronous cells (Fig. 3A). Release of the cells from nocodazole-induced arrest resulted both in the rapid degradation of cyclin B1 and securin (Fig. 3A) and in exit of the cells from mitosis and their progression into G_1 phase (Fig. 3B). However, the release of arrested cells in the presence of H_2O_2 prevented the destruction of cyclin B1 and securin (Fig. 3A) as well as delayed cell cycle progression (Fig. 3B) in a concentration-dependent manner. These results suggested that the machinery responsible for the degradation of cyclin B1 and securin in mitotic cells is inhibited by H_2O_2.

To test whether H_2O_2 affected the ubiquitination of APC substrates, we measured the ubiquitination of cyclin B1 in HeLa cells expressing HA-tagged ubiquitin. Release of the cells from mitotic arrest in the presence of the proteasome inhibitor MG-132 resulted in the appearance of multiple ubiquitinated bands in immunoblots of cyclin B1 immunoprecipitates probed with antibodies to HA (Fig. 3C). The presence of H_2O_2 in the release medium inhibited the ubiquitination of cyclin B1, suggesting that H_2O_2 blocks the degradation of APC substrates by inhibiting the ubiquitin ligase activity of APC.

Ubc4 interacts directly with APC11 and thereby supports the ubiquitination reaction [20–22,33]. To determine whether the APC11–Ubc4 interaction was also inhibited by H_2O_2, we expressed HA-tagged APC11 together with Flag-tagged Ubc4 in HeLa cells, immunoprecipitated HA-APC11 with antibodies to HA, and subjected the immunoprecipitates to immunoblot analysis with antibodies to Flag. Flag-Ubc4 coprecipitated in similar amounts with HA-APC11 from cells arrested in mitosis or released from such arrest (Fig. 3D). The release of arrested cells in the presence of H_2O_2, however, resulted in a marked decrease in the amount of coprecipitated Flag-Ubc4.

H_2O_2 oxidizes cysteine residues of APC11 in vivo

To investigate whether the cysteine residues of APC11 are oxidized in HeLa cells treated with H_2O_2, we applied a thiol-trapping method that allows the separation of oxi-

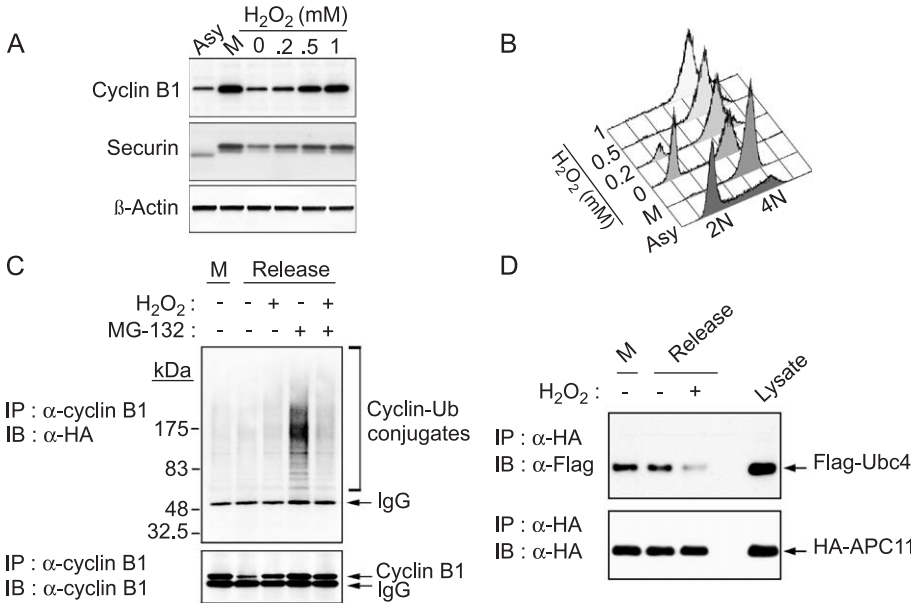

Fig. 3. Inhibition of APC function in HeLa cells by H_2O_2. (A) Prevention by H_2O_2 of the degradation of APC substrates. HeLa cells were synchronized in metaphase by nocodazole treatment and then released from arrest and incubated for 90 min with the indicated concentrations of H_2O_2. Lysates prepared from asynchronous (Asy), metaphase-arrested (M), and released cells were subjected to immunoblot analysis with antibodies to cyclin B1, to securin, or to β-actin (control). (B) Delay in cell cycle progression into G_1 phase induced by H_2O_2. The cells studied in (A) were also subjected to flow cytometric analysis of DNA content. Relative cell number is plotted against DNA content per cell. (C) Inhibition by H_2O_2 of cyclin B1 ubiquitination. HeLa cells that had been transfected for 36 h with pHM6-ubiquitin encoding HA-ubiquitin were synchronized in metaphase, released from cell cycle arrest, and incubated for 90 min in the absence or presence of 0.5 mM H_2O_2 or 50 μM MG-132, as indicated. Cell lysates prepared from the metaphase-arrested (M) or released cells were subjected to immunoprecipitation (IP) for 6 h at 4 °C with agarose–conjugated antibodies to cyclin B1 (α-cyclin B1). The resulting precipitates were then subjected to immunoblot analysis (IB) with antibodies to HA (upper panel), after which the membrane was reprobed with antibodies to cyclin B1 (lower panel). The positions of cyclin B1–ubiquitin conjugates, cyclin B1, and IgG heavy chain are indicated on the right. (D) Inhibition by H_2O_2 of the interaction between APC11 and Ubc4. HeLa cells that had been cotransfected for 36 h with pCGN-APC11 (1 μg) and pCMV2B-Ubc4 (3 μg), which encode HA-APC11 and Flag-Ubc4, respectively, were synchronized in metaphase, released from cell cycle arrest, and incubated for 45 min in the absence or presence of 0.5 mM H_2O_2. Cell lysates prepared from metaphase-arrested (M) or released cells were incubated at 4 °C first for 4 h with antibodies to HA and then for 1 h in the additional presence of protein G–Sepharose beads. The resulting immunoprecipitates as well as the lysate of the mitotically arrested cells (50 μg of protein) were subjected to immunoblot analysis with antibodies to Flag (upper panel), after which the membrane was reprobed with antibodies to HA (lower panel). The positions of Flag-Ubc4 and HA-APC11 are indicated. Data in (A) through (D) are representative of three separate experiments.

dized and reduced species on nonreducing gels [34,37]. All accessible thiol groups in cell lysates were alkylated irreversibly with NEM, after which disulfide bonds present in proteins were reduced to thiols with DTT. The newly produced thiol groups were then alkylated with AMS, a thiol-reactive reagent that alkylates cysteine residues and thereby adds 500 Da to each thiol group.

HeLa cells expressing HA-APC11 were subjected to mitotic arrest and then released for 60 min in the presence of various concentrations of H_2O_2. Cell lysates were prepared and subjected to the thiol-trapping procedure, and any change in the size of HA-APC11 in the lysates was detected by SDS–PAGE under nonreducing conditions followed by immunoblot analysis with antibodies to HA (Fig. 4A). Treatment of the cells with H_2O_2 induced a concentration-dependent increase in the amount of oxidized (slower migrating) APC11. To determine whether the disulfide bonds formed by APC11 might be intersubunit, as observed with recombinant APC11 in vitro (Fig. 2B), we subjected the cell lysates to nonreducing SDS–PAGE and immunoblot analysis without subjecting them to the thiol-trapping procedure. HA-APC11 was detected as a monomer in lysates derived from cells treated or not with H_2O_2 (data not shown), indicating that the cysteine residues of APC11 form intramolecular disulfides when the protein is assembled in a complex with other APC subunits.

Mammalian cells contain various peroxidases and catalases that are able to metabolize H_2O_2. Thus, H_2O_2 added as a bolus at various levels to the culture medium of HeLa cells was gradually depleted, with a half-time of ~25 min when added at 1 mM (Fig. 4B). It was therefore possible that both the continuous decay of H_2O_2 and the slow release of Zn^{2+} might be responsible for the relatively high initial concentrations of exogenous H_2O_2 required for the oxidation of APC11 in cells.

To determine whether low steady-state levels of H_2O_2 could be achieved with the use of glucose oxidase, which

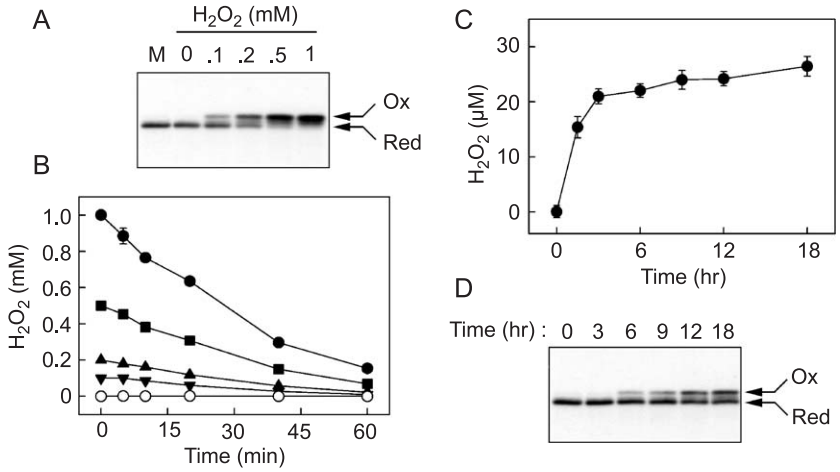

Fig. 4. Oxidation of cysteines of APC11 induced by H_2O_2 in HeLa cells. (A) Oxidation of APC11 in vivo. HeLa cells that had been transfected with 1 μg of pCGN-APC11 encoding HA-APC11 for 36 h were synchronized in metaphase by nocodazole treatment, released from arrest, and incubated for 60 min in the presence of the indicated concentrations of H_2O_2. Lysates of the metaphase-arrested cells (M) and those of cells released in the presence of H_2O_2 were subjected to a thiol-trapping procedure to allow separation of reduced and oxidized forms of HA-APC11, as described under Methods. The samples were then subjected to nonreducing SDS–PAGE followed by immunoblot analysis with antibodies to HA. The positions of the reduced and oxidized proteins are indicated. (B) Depletion of exogenous H_2O_2 by HeLa cells. The concentration of H_2O_2 remaining in the culture medium of HeLa cells was measured at the indicated times after the addition of various initial concentrations (○, 0; ▼, 0.1; ▲, 0.2; ■, 0.5; and ●, 1 mM). (C) Continuous production of H_2O_2 in culture medium by glucose oxidase. HeLa cells (3×10^5 cells in a 60-mm dish) that had been transiently transfected with pCGN-APC11 were incubated in medium containing glucose oxidase (7.5 mU/ml). The concentration of H_2O_2 in the medium was determined at the indicated times. (D) Oxidation of APC11 in HeLa cells exposed to a steady-state level of H_2O_2. After exposure to the glucose oxidase–containing medium for the indicated times, the cells from the experiment shown in (C) were analyzed for the reduced and oxidized forms of HA-APC11 as described in (A). Data in (A) through (D) are representative of three separate experiments.

catalyzes the oxidation of β-D-glucose in the presence of oxygen to produce D-gluconic acid and H_2O_2, we incubated HeLa cells with various concentrations of this enzyme and measured the concentration of H_2O_2 in the medium. In the presence of glucose oxidase at a concentration of 7.5 mU/ml, the concentration of H_2O_2 reached a plateau after 3 h and was then maintained in the range of 20 to 30 μM as the result of the balance between consumption by the cells and production by glucose oxidase (Fig. 4C).

We then determined the oxidation state of APC11 in HeLa cells exposed to the steady-state level (20 to 30 μM) of H_2O_2 (Fig. 4D). Incubation of cells with glucose oxidase resulted in a slow oxidation of APC11, with ~50% of APC11 in the oxidized form after 18 h. These results thus suggest that the low levels of H_2O_2 produced under certain pathophysiological conditions might be sufficient to inhibit the ubiquitination activity of APC11.

DISCUSSION

Hydrogen peroxide is a mild oxidant that is able to oxidize cysteine residues in proteins to cysteine sulfenic acid or disulfide, both of which are readily reduced back to cysteine by various cellular reductants. Because the pK_a (where K_a is the acid constant) of the sulfhydryl group (Cys–SH) of most cysteine residues is ~8.5, and because this group is less readily oxidized by H_2O_2 than is the cysteine thiolate anion ($Cys–S^-$), few proteins might be expected to possess a Cys–SH that is vulnerable to oxidation by H_2O_2 in cells. However, certain protein cysteine residues do exist as thiolate anions at neutral pH either as a result of the lowering of their pK_a values by charge interactions between the negatively charged thiolate and nearby positively charged amino acid residues or as a result of being a ligand of a metal ion such as Zn^{2+}.

The displacement of 50% of bound zinc from APC11 required ~30 min in the presence of H_2O_2 at a concentration of 0.5 mM. This rate is similar to those observed with other zinc-containing proteins such as metallothionein (half-time of ~20 min in the presence of 1 mM H_2O_2) [48], heat shock protein 33 (half–time of ~10 min in the presence of 3 mM H_2O_2) [34], and the retinoic acid receptor (half–time of ~30 min in the presence of 1 mM H_2O_2) [38]. The release of zinc associated with proteins is thus a relatively slow process. Although, like other cysteine thiolate groups, zinc-coordinated thiolates might be expected to react rapidly with H_2O_2, the slow rate of Zn^{2+} release is probably due to the fact that Zn^{2+} can remain bound through the remaining three ligands after dissociation of one oxidized cysteine. Our results indicate that APC11 binds Zn^{2+} at a molar ratio of 1:3, consistent with the previous demonstration that APC11

contains a third zinc binding site in addition to the two formed by the canonical RING-H2 finger [22].

In addition to maintaining the structural integrity of the RING finger, Zn^{2+} was suggested to participate directly in catalysis [22]. Unlike the two zinc ions of the RING-finger motif, the binding of the third zinc ion was found not to be important for the E3 activity of APC11 [22]. Our results indicate that H_2O_2 both induces Zn^{2+} release from APC11 by oxidizing the coordinating cysteine residues and prevents the binding of APC11 to the E2 enzyme Ubc4 both in vitro and in vivo.

Hydrogen peroxide induces a variety of cellular responses that depend on its concentration. At concentrations of ~1 to 15 μM, depending on cell type, H_2O_2 serves as a mitogenic signal [49–54]. It thus induces quiescent cells to enter the cell cycle [51], and this effect is accompanied by signaling events such as protein tyrosine phosphorylation and early gene expression. The mitogenic effect of peptide growth factors is also accompanied by the intracellular production of H_2O_2, and such receptor-mediated mitogenic signaling is abrogated by prevention of the accumulation of H_2O_2 with catalase [55]. An important target for the H_2O_2 produced in response to growth factors or for exogenous H_2O_2 present at low concentrations appears to be the active site cysteine of protein tyrosine phosphatases and of PTEN, a phosphoinositide 3-phosphatase [56,57]. It has thus been proposed that the activation of protein tyrosine kinases and phosphoinositide 3-kinase by growth factors might not be sufficient to increase the steady-state levels of protein tyrosine phosphorylation and 3-phosphorylated phosphoinositides in cells and that concurrent inhibition of protein tyrosine phosphatases and PTEN by H_2O_2 is also needed.

Exposure of dividing cells to concentrations of H_2O_2 (~ 100 to 200 μM) higher than those that induce a mitogenic response results in temporary growth arrest [50,53,54,58–60]. During this growth arrest, the expression of antioxidant enzymes, damage-repair enzymes, and shock-response proteins is increased, whereas that of many housekeeping enzymes is reduced. Cell cycle progression is resumed after the removal of H_2O_2 and the repair of damaged DNA. The temporary arrest thus provides cells with the time needed for defense against and recovery from the oxidative stress. Exposure of cells to H_2O_2 at even higher concentrations or for prolonged times induces permanent growth arrest, which is phenotypically similar to replicative senescence [54,61,62]. At concentrations in the range of 0.5 to 5 mM, H_2O_2 induces apoptosis or necrosis [50,63–70].

Cell cycle arrest has been associated with the activation of p53 and modified expression of several proteins, including gadd153, gadd45, adapt15, and $p21^{Cip1}$ [50,58]. Our results now suggest that the direct oxidation of APC11 can result in cell cycle arrest (probably either temporary or permanent) in G_2-M phase. The arrest of oxidatively stressed cells in mitosis has previously been described [71–73]. Because the SCF-type E3 complex, which functions at the G_1–S transition, also contains a RING-finger protein, Rbx1 (ROC1), H_2O_2 also likely contributes to growth arrest by inhibiting cell cycle progression through G_1. The thiolate anions coordinated by zinc, such as those in APC11 and several other zinc-finger proteins, are less sensitive to oxidation by H_2O_2 than are the active site thiolate anions of protein tyrosine phosphatases. Thus, whereas treatment of HeLa cells for 30 min with 20 μM H_2O_2 or EGF elicited a strong tyrosine phosphorylation of several proteins, the same treatments did not induce oxidation of APC11 when measured using the thiol-trapping method (not shown). This differential sensitivity to oxidation may provide cells with a mechanism for distinguishing between levels of H_2O_2 that promote mitosis and those that are toxic. Our observation that prolonged exposure of cells to relatively low levels of H_2O_2 (20 to 30 μM) was sufficient to induce oxidation of APC11 suggests that oxidative stress associated with pathological conditions such as chronic inflammation also might be able to induce cell cycle arrest by inhibiting APC function.

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ABBREVIATIONS

AMS — 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate
APC — anaphase-promoting complex
HA — hemagglutinin epitope
NEM — *N*-ethylmaleimide
PAGE — polyacrylamide gel electrophoresis
PAR — 4-(2-pyridylazo) resorcinol
PMPS — *p*-hydroxymercuriphenylsulfonic acid
TCA — trichloroacetic acid