Cooperation of HECT-domain Ubiquitin Ligase hHYD and DNA Topoisomerase II-binding Protein for DNA Damage Response*

Yoshiomi Honda‡‡, Masahide Tojo‡, Kazuhiro Matsuzaki‡, Tadashi Anan‡, Mitsuhiro Matsumoto‡, Masayuki Ando‡, Hideyuki Saya‡, and Mitsuyoshi Nakao‡‡

From the ‡Department of Tumor Genetics and Biology and ¶Department of Internal Medicine I, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan

Ubiquitin ligases define the substrate specificity of protein ubiquitination and subsequent proteosomal degradation. The catalytic sequence was first characterized in the C terminus of E6-associated protein (E6AP) and referred to as the HECT (homologous to E6AP C terminus) domain. The human homologue of the regulator of cell proliferation hyperplastic discs in Drosophila, designated hHYD, is a HECT-domain ubiquitin ligase. Here we show that hHYD provides a ubiquitin system for a cellular response to DNA damage. A yeast two-hybrid screen showed that DNA topoisomerase II-binding protein 1 (TopBP1) interacted with hHYD. Endogenous hHYD bound the BRCA1 C-terminus domains of TopBP1 that are highlighted in DNA damage checkpoint proteins and cell cycle regulators. Using an in vitro reconstitution, specific E2 (ubiquitin-conjugating) enzymes (human UbcH4, UbcH5B, and UbcH5C) transferred ubiquitin molecules to hHYD, leading to the ubiquitination of TopBP1. TopBP1 was usually ubiquitinated and degraded by the proteosome, whereas X-irradiation diminished the ubiquitination of TopBP1 probably via the phosphorylation, resulting in the stable colocalization of up-regulated TopBP1 with γ-H2AX nuclear foci in DNA breaks. These results demonstrated that hHYD coordinated TopBP1 in the DNA damage response.

The ubiquitin-proteosome proteolytic system is involved in a variety of fundamental cell regulations including gene expression, stress response, DNA repair, and cell cycles (1–6). Ubiquitination includes a cascade of three classes of enzymes, the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme Ubc or E2, and the ubiquitin ligase E3. After activation of a ubiquitin by E1, E2 and E3 cooperate to catalyze the formation of a mult ubiquitin chain on a protein substrate. The E3 enzymes or the protein complexes with a ligase activity are believed to target the substrate for selective ubiquitination and a subsequent turnover by a large protease complex, the 26 S proteosome. There are two distinct groups of human ubiquitin ligases, the HECT domain E3 enzymes, including E6-associated protein (E6AP) and NEDD4, and the RING finger E3 enzymes, including SCF (Skp1-cullin-F box), VBC (VHL-Elongin B-Elongin C), APC (anaphase-promoting complex) and other single RING finger proteins such as c-Cbl and MDM2 (7). The HECT sequence of ~200 amino acids is highly conserved in the C-terminal catalytic domain of family members from yeast to mammals. The HECT-domain protein forms a thiol ester bond with a ubiquitin at the active cysteine residue and transfers the ubiquitin directly to the substrate (8–10), whereas RING finger proteins form complexes containing an E2 enzyme, which facilitate ubiquitination of the substrate. However, the overall mechanisms by which these ubiquitin ligases recognize the target proteins remain to be elucidated.

In the present study, we focused on characterizing the human HECT-domain protein, a counterpart of the regulator of cell proliferation and the putative tumor suppressor hyperplastic discs in Drosophila melanogaster, termed hHYD. The hyperplastic discs gene was initially cloned by investigating temperature-sensitive mutants that caused imaginal disc over-growth in larvae at restrictive temperatures (11, 12). Recently, hHYD, also named EDD, was reported to be transcriptionally induced by progesterin in human breast cancer cells, suggesting a role for hHYD in hormone-mediated cell regulation in higher eukaryotes (13). Here, we identified that hHYD interacts with DNA topoisomerase-IIβ-binding protein 1 (TopBP1) (14, 15). Interestingly, TopBP1 shows the presence of BRCA1 C terminus (BRCT) domains, which are found in many molecules involved in DNA damage response and checkpoint-mediated repair including BRCA1, p53-binding protein 1 (53BP1), Nijmegen breakage syndrome 1 (NBS1), x-ray repair complementing 1 (XRCC1), DNA ligases 3 and 4, and poly-(ADP-ribose) polymerase (PARP) (16–19). Biochemically, specific E2 enzymes transferred ubiquitin molecules to hHYD, resulting in the ubiquitination of TopBP1. TopBP1 was usually ubiquitinated and degraded by the proteosome, whereas X-irradiation reduced the ubiquitination of TopBP1 via the phosphorylation, causing the colocalization of TopBP1 with γ-H2AX in DNA break sites. Our observations demonstrated that hHYD regulates TopBP1 for the DNA damage response.

EXPERIMENTAL PROCEDURES

Cloning of Human HYD—We performed a tblastn data base search using the amino acid sequences of the hyd in Drosophila and found several human EST clones homologous to the sequences. Using human cDNA libraries and cDNA fragments, PCR amplifications were performed to isolate full-length hHYD. We identified a 8397-base pair cDNA that contained a single large open reading frame encoding a...
Ubiquitination of TopBP1 by hHYD

polypeptide of 2798 amino acid residues (GenBankTM accession number U95000). The hHYD showed sequences similar to those of EDD (GenBankTM accession number AF006010).

Yeast Two-hybrid Screen—Yeast strain L40 carrying pBTM116HA-hHYD (amino acids 2520–2798) was transformed with the HeLa and skeletal muscle cDNA libraries constructed in pGAD-GH (CLONTECH) utilizing the region of amino acids 2520–2798, which contains the HECT domain and its N-terminal portion of hHYD as bait (Fig. 1A). From a screening of ~12 × 10⁶ independent transformants, 10 clones were β-galactosidase-positive on histidine-deficient plates. From the positive clones, we isolated 2 independent cDNA clones encoding the C-terminal region of TopBP1 (amino acids 645–1522). Yeast cells expressing both hHYD and TopBP1 grew selectively on plate media lacking tryptophan, leucine, and histidine (Fig. 1B). TopBP1 possessed eight tandem repeats of the BRCT domain. DNA topoisomerase IIβ, which catalyzes transient breaks and topological changes of DNA for replication and chromosome segregation, was originally reported to bind the C-terminal half of TopBP1 (amino acids 862–1522) (15). To examine whether TopBP1 specifically interacts with hHYD, FLAG-tagged TopBP1 was expressed in COS-7 cells for immunoprecipitation with rabbit anti-hHYD and anti-NEDD4 polyclonal antibodies. NEDD4 is another member of the HECT-domain ligases with distinct features in the N-terminal region of the protein (20, 22). Western blot analysis using an anti-FLAG antibody revealed that overexpressed TopBP1 was detected in the hHYD- but not in the NEDD4-immunoprecipitates (Fig. 1C). Both endogenous hHYD and NEDD4 were present in these immunoprecipitates (data not shown). To determine the region of TopBP1 interacting with hHYD, we constructed two C-terminal deletion mutants of FLAG-TopBP1, Δ1 and Δ2, containing the regions of amino acids 1–1080 and 1–660, respectively (Fig. 1A). Native hHYD was immunoprecipitated with anti-hHYD antibodies from FLAG-TopBP1-expressing cells, and an anti-FLAG antibody detected each of the FLAG-TopBP1 proteins (Fig. 1D). Full-length TopBP1 and the Δ1 mutant were coprecipitated with hHYD (lanes 2 and 4), whereas the Δ2 mutant was scarcely found in the hHYD immunoprecipitate (lane 6). Approximately 0.5% of overexpressed full-length TopBP1 in the input lysate was expected to associate with hHYD. The FLAG-TopBP1 proteins were equally expressed before immunoprecipitation (lanes 1, 3, and 5). Collectively, hHYD interacts with the minimal region of the amino acids 661–1080 including BRCT5 and BRCT6 of TopBP1, suggesting a specific interaction of hHYD and TopBP1. In Fig. 1E, we show expression plasmids for hHYD and TopBP1 in COS-7 cells along with affinity-purified rabbit polyclonal antibodies against recombinant hHYD and TopBP1 proteins. Western blot analysis showed that FLAG-tagged hHYD and TopBP1 were detected using appropriate antibodies in the transfected cells (lanes 2 and 4, and lanes 8 and 10, respectively). In addition, the anti-hHYD antibodies cross-reacted with endogenous protein (lane 3), whereas the anti-TopBP1 antibodies contributed few bands in the mock-transfected cells (lane 9), probably due to the low levels of native TopBP1 in intact cells.

In Vitro Ubiquitination of TopBP1 by E1-E2-hHYD Cascade—To identify the E1-E2-hHYD pathway, we first analyzed in vitro ubiquitin transfer to glutathione S-transferase (GST)-fused hHYD in 50 mm Tris-HCl (pH 8.3), 5 mm MgCl₂, 1.6 mm ATP, 2 mm ATP-γ-S, and 1 mm dithiothreitol at 30 min at 25 °C. Reactions were terminated by incubating the mixtures for 20 min at 25 °C in a sample buffer and electrophoresed by SDS-PAGE.

RESULTS

Interaction of hHYD with TopBP1—To identify a protein factor that interacts with hHYD, we performed a yeast two-hybrid screen of HeLa and skeletal muscle cDNA libraries utilizing the region of amino acids 2520–2798, which contains the HECT domain and its N-terminal portion of hHYD as bait (Fig. 1A). From a screening of ~12 × 10⁶ independent transformants, 10 clones were β-galactosidase-positive on histidine-deficient plates. From the positive clones, we isolated 2 independent cDNA clones encoding the C-terminal region of TopBP1 (amino acids 645–1522). Yeast cells expressing both hHYD and TopBP1 grew selectively on plate media lacking tryptophan, leucine, and histidine (Fig. 1B). TopBP1 possessed eight tandem repeats of the BRCT domain. DNA topoisomerase IIβ, which catalyzes transient breaks and topological changes of DNA for replication and chromosome segregation, was originally reported to bind the C-terminal half of TopBP1 (amino acids 862–1522) (15). To examine whether TopBP1 specifically interacts with hHYD, FLAG-tagged TopBP1 was expressed in COS-7 cells for immunoprecipitation with rabbit anti-hHYD and anti-NEDD4 polyclonal antibodies. NEDD4 is another member of the HECT-domain ligases with distinct features in the N-terminal region of the protein (20, 22). Western blot analysis using an anti-FLAG antibody revealed that overexpressed TopBP1 was detected in the hHYD- but not in the NEDD4-immunoprecipitates (Fig. 1C). Both endogenous hHYD and NEDD4 were present in these immunoprecipitates (data not shown). To determine the region of TopBP1 interacting with hHYD, we constructed two C-terminal deletion mutants of FLAG-TopBP1, Δ1 and Δ2, containing the regions of amino acids 1–1080 and 1–660, respectively (Fig. 1A). Native hHYD was immunoprecipitated with anti-hHYD antibodies from FLAG-TopBP1-expressing cells, and an anti-FLAG antibody detected each of the FLAG-TopBP1 proteins (Fig. 1D). Full-length TopBP1 and the Δ1 mutant were coprecipitated with hHYD (lanes 2 and 4), whereas the Δ2 mutant was scarcely found in the hHYD immunoprecipitate (lane 6). Approximately 0.5% of overexpressed full-length TopBP1 in the input lysate was expected to associate with hHYD. The FLAG-TopBP1 proteins were equally expressed before immunoprecipitation (lanes 1, 3, and 5). Collectively, hHYD interacts with the minimal region of the amino acids 661–1080 including BRCT5 and BRCT6 of TopBP1, suggesting a specific interaction of hHYD and TopBP1. In Fig. 1E, we show expression plasmids for hHYD and TopBP1 in COS-7 cells along with affinity-purified rabbit polyclonal antibodies against recombinant hHYD and TopBP1 proteins. Western blot analysis showed that FLAG-tagged hHYD and TopBP1 were detected using appropriate antibodies in the transfected cells (lanes 2 and 4, and lanes 8 and 10, respectively). In addition, the anti-hHYD antibodies cross-reacted with endogenous protein (lane 3), whereas the anti-TopBP1 antibodies contributed few bands in the mock-transfected cells (lane 9), probably due to the low levels of native TopBP1 in intact cells.

In Vitro Ubiquitination of TopBP1 by E1-E2-hHYD Cascade—To identify the E1-E2-hHYD pathway, we first analyzed in vitro ubiquitin transfer to glutathione S-transferase (GST)-fused hHYD in 50 mm Tris-HCl (pH 8.3), 5 mm MgCl₂, 1.6 mm ATP, 2 mm ATP-γ-S, and 1 mm dithiothreitol at 30 min at 25 °C. Reactions were terminated by incubating the mixtures for 20 min at 25 °C in a sample buffer and electrophoresed by SDS-PAGE.

Immunoprecipitation and α-Phosphatase Treatment—The cells were lysed in a lysis buffer (1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mm 4-(2-aminoethyl)-benzenesulfonil fluoride hydrochloride, 3% aprotinin, 10 μg/ml leupeptin, 1.5 μM pepstatin, 1 mm sodium orthovanadate in PBS (pH 8.0)). An appropriate antibody was added to the lysate and incubated for 8 h. The immunocomplexes were collected with protein (G–A)-conjugated agarose and washed with a buffer (25 mm Tris-HCl (pH 8.0), 150 mm NaCl, 0.5% Nonidet P-40) (21). Immunoprecipitated proteins were separated on 3–10% gradient polyacrylamide gel. For the phosphorylation assay, immunoprecipitated proteins were incubated with α protein phosphatase (New England BioLabs) at 30°C for 1 h.

Immunofluorescence Microscopy—The fibroblasts were washed 2 times with PBS and fixed in 4% paraformaldehyde for 10 min followed by 0.2% Triton X-100 for 5 min. After washing with PBS, the cells were incubated with diluted anti-hHYD, anti-TopBP1 and anti-γ-H2AX antibodies in PBS containing 0.2% bovine serum albumin for 60 min at room temperature. After washing with PBS, the cells were incubated with Cy3- and fluorescein isothiocyanate-linked secondary antibodies (Amersham Biosciences, Inc.) for 60 min. After washing again with PBS, the cells were mounted on 80% glycerol and visualized with a confocal laser-scanning microscope.

In Vitro Ubiquitination Assay—Ubiquitin transfer and conjugation were performed as reported previously (20). Reaction mixtures contained ~0.15 μg of E1, 0.5 μg of each E2, 1.0 μg of bacterially expressed hHYD or baculovirus-expressed hHYD, and 0.5 μg of Myc-tagged ubiquitin in 50 mm Tris-HCl (pH 8.3), 5 mm MgCl₂, 1.6 mm ATP, 2 mm ATP-γ-S, and 1 mm dithiothreitol for 30 min at 25 °C. Reactions were

http://www.jbc.org/
ence of E1 and UbcH4. In contrast, the addition of dithiothreitol diminished the intensity of the bands corresponding to UbcH4-ubiquitin and hHYD-ubiquitin conjugates. The E1-ubiquitin seemed to be hardly visible in the gel. Neither E1 nor UbcH4 alone transferred ubiquitin to hHYD. The appearance of two close-migrating bands of hHYD-ubiquitin might have represented hHYD conjugated with ubiquitins as with other HECT proteins (8, 20), and Huibregtse et al. (8) suggest an autoubiquitination or an intramolecular transfer of ubiquitins. The magnification showed the relative locations of hHYD and hHYD-ubiquitins on reprobing the same membrane with anti-hHYD antibodies (lower panel). To determine the E2 enzymes coupled to hHYD, we utilized nine kinds of recombinant E2 proteins in the presence of E1 and hHYD. hHYD accepted ubiquitin molecules especially from UbcH4, UbcH5B, and UbcH5C and weakly from UbcH7 (data not shown). We next constituted the E1-E2-hHYD cascade using a baculovirus-expressed full-length hHYD (Fig. 2B). For in vitro ubiquitination, E1, full-length hHYD, Myc-ubiquitin, and the C-terminal region of TopBP1 were incubated with each of the E2 enzymes, and the state of TopBP1 was examined with anti-TopBP1 and anti-Myc antibodies. The combinations of hHYD with UbcH4, UbcH5B, and UbcH5C strongly induced the ubiquitination of TopBP1. In addition, TopBP1 was weakly ubiquitinated in the presence of UbcH2A (HHR6A),

---

**Fig. 1. Interaction of hHYD with TopBP1.**

**A**, structures of hHYD and TopBP1. The hHYD represents a putative nuclear localization signal (NLS), a region homologous to poly(A)-binding proteins (PABP), and the C-terminal HECT ubiquitin-ligase domain. TopBP1 has eight repeated BRCT domains and a putative nuclear localization signal (15). Two arrows indicate the binding regions identified in the yeast two-hybrid screen. The C-terminal deletion mutants of TopBP1, Δ1 and Δ2, are also shown. **B**, specific interaction between hHYD and TopBP1 in yeast L40 cells on a plate lacking tryptophan, leucine, and histidine. **C**, presence of TopBP1 in hHYD immune complexes. Another HECT-domain protein, NEDD4, did not associate with TopBP1. **IP**, immunoprecipitates. **D**, region interacting with hHYD in TopBP1. Native hHYD was immunoprecipitated with the anti-hHYD antibodies from the FLAG-TopBP1-transfected COS-7 cells. **E**, expression of hHYD and TopBP1 in mammalian cells and affinity-purified rabbit anti-hHYD and anti-TopBP1 polyclonal antibodies. For the Western blot analysis, mock plasmid and preimmune serum (Preim.) were used as a control.
Fig. 2. *In vitro* ubiquitination of TopBP1 by E1-E2-hHYD cascades. A, the combination of E1 and UbcH4 efficiently transfers ubiquitins (Ub) to GST-fused hHYD (residues 2520–2798). The results are shown in the presence and the absence of dithiothreitol (DTT). The lower panel indicates the magnification of GST-hHYD and GST-hHYD associated with ubiquitins. B, ubiquitination of TopBP1 by baculovirus-expressed full-length hHYD in the presence of E1 and E2 enzymes. hHYD ubiquitinates TopBP1 in cooperation with specific E2 enzymes such as UbcH4, HbcH5B, and UbcH5C. C, inhibition of TopBP1 ubiquitination by preincubating with anti-hHYD and anti-TopBP1 antibodies. Control and heat-inactivated antibodies were utilized. Reactions contained bacterially expressed Myc-ubiquitin, E1, each of the E2 enzymes, hHYD, the C-terminal half of TopBP1, and ATP as described under “Experimental Procedures.” The E1-ubiquitin, E2-ubiquitin, hHYD-ubiquitin, and ubiquitinated TopBP1 are analyzed by anti-Myc and anti-TopBP1 antibodies. Preim., preimmune serum.
Ubiquitination of TopBP1 by hHYD

Ubiquitination of TopBP1 by hHYD—To confirm the ubiquitin ligase activity of hHYD in the cells, we expressed FLAG-tagged, wild- and mutant-type hHYD together with HA-tagged ubiquitin. The mutant hHYD(C/A) substituted the active site cysteine with alanine at amino acid 2767. hHYD was immunoprecipitated with the anti-FLAG antibody from the transfected cells, and the immune complexes were probed with an anti-HA antibody (Fig. 3A). The anti-HA reactive, high molecular mass proteins were detected as ubiquitinated forms of the substrates (lane 2). The use of proteosome inhibitor MG132 resulted in the accumulation of multiple ubiquitinated proteins (lane 3), indicating that these ubiquitinated proteins were naturally degraded by the proteosome. In contrast, ubiquitinated proteins were only scantily coprecipitated with the mutant hHYD (lane 4), demonstrating that hHYD normally ubiquitinates cellular proteins. FLAG-hHYD was similarly immunoprecipitated (lanes 2–4). To directly clarify the ubiquitination of TopBP1, both FLAG-TopBP1 and HA-ubiquitin were expressed in COS-7 cells. TopBP1 was immunoprecipitated with the anti-FLAG antibodies, and the immunoprecipitates, after frequent washings, were detected with anti-HA antibodies (Fig. 3B). High molecular mass ubiquitinated TopBP1 appeared in the upper part of the unconjugated form (lane 5), and the proteosome inhibition by MG132 markedly accumulated the ubiquitinated proteins (lane 6). Mock transfection lacked such ubiquitinated bands (lanes 4). A slight decrease of TopBP1 itself in the MG132-treated input lysate might reflect the considerable increase of the ubiquitinated forms rather than TopBP1 itself (lanes 3 and 6). Taken together, these results showed that TopBP1 is ubiquitinated in the cells as well as in vitro.

X-irradiation Reduced TopBP1 Ubiquitination via Phosphorylation—To further conclude the multiple ubiquitination of TopBP1 in the cells, we expressed both FLAG-TopBP1 and HA-ubiquitin in COS-7 cells. Immunoprecipitates with the anti-HA antibodies were probed with anti-TopBP1 antibodies (Fig. 4A), providing the evidence for the ubiquitinated and some degraded TopBP1 (lane 6). The proteosome inhibition by MG132 markedly augmented the ubiquitinated TopBP1 (lane 7). Mock transfection showed no bands that cross-reacted to the anti-TopBP1 antibodies (lane 5). Next, the fact that BRCT-domain proteins are involved in DNA damage response and cell cycle checkpoint prompted us to test whether X-irradiation affects the TopBP1 ubiquitination (lane 8). Despite the presence of MG132, it is of great interest that irradiation (12 Gy) strongly diminished the ubiquitinated TopBP1. Furthermore, we then analyzed the ubiquitination of endogenous TopBP1 after DNA damage in U2OS cells that have been proved to properly respond to various DNA damages. TopBP1 was immunoprecipitated and detected with the anti-TopBP1 antibodies (Fig. 4B). There were the high molecular bands corresponding to TopBP1 in addition to the unconjugated form under the proteosome inhibition by MG132 (lane 2). As was the case in COS-7 cells, irradiation (12 Gy) dramatically reduced the probably ubiquitinated forms of TopBP1 (lane 3). We did not find such conjugated forms of the protein in the control (lane 1). In addition, unconjugated TopBP1 tended to be slow migrating in lane 3. Because BRCT-domain proteins are mostly phosphorylated under DNA damage, we investigated whether irradiation (12 Gy) causes the phosphorylation of TopBP1 (Fig. 4C). Irradiation-induced mobility shift of TopBP1 was found in lane 2, and it was restored to the original state similar to the control by a protein phosphatase (lane 3). The amount of TopBP1 seemed to increase in the damaged cells. Immunoprecipitation with preimmune antibodies gave no such bands (lanes 4–6). Taken together, these suggested that DNA double strand breaks induce the phosphorylated form of TopBP1 and inhibit the TopBP1 ubiquitination in the cells.

Colocalization of TopBP1 with γ-H2AX in Irradiated Human Fibroblasts—We visualized the localization of cellular hHYD and TopBP1 in immortalized human fibroblasts. A confocal laser-scanning microscopic analysis of the intact and X-irradiated cells was performed using anti-hHYD and anti-TopBP1 antibodies.
antibodies (Fig. 4D). Both TopBP1 and hHYD formed fine speckles in the nucleus of the intact cells (a and b). Although the TopBP1 foci were very small, the fraction of TopBP1 appeared to associate with hHYD (c). Interestingly, TopBP1 formed multiple discrete foci in the nucleus at 8 h after irradiation (12 Gy) (d), whereas a number of hHYD foci were detected in the irradiated cells (e). TopBP1 did not specifically coexist with hHYD in the damaged cells (f). To further investigate the

**Fig. 4.** TopBP1 in X-irradiation-induced DNA damage response. 

A. Inhibition of TopBP1 ubiquitination by X-irradiation. Both FLAG-TopBP1 and HA-ubiquitin were expressed in COS-7 cells, and the HA immunoprecipitates (IP) were probed with anti-TopBP1 antibodies. The cells were irradiated (IR; 12 Gy), and proteosome inhibitor MG132 (10 μM) was then added for 8 h. B, loss of high molecular forms of endogenous TopBP1 under DNA damage. TopBP1 in U2OS cells was immunoprecipitated and detected with anti-TopBP1 antibodies. The proteosome inhibition and irradiation were performed as above. C, phosphorylation of TopBP1 in X-irradiation. Irradiation induced the mobility shift of TopBP1, which is sensitive to λ-phosphatase (λPase). The phosphorylated and unphosphorylated forms of TopBP1 are indicated by open and closed circles, respectively. D, irradiation-induced foci formation of up-regulated TopBP1 in the nucleus. Affinity-purified antibodies counterstained cellular TopBP1 (red) and hHYD (green). E, colocalization of TopBP1 (red) and γ-H2AX (green) in damaged DNA sites. Human fibroblasts were irradiated (12 Gy), resulting in the complex formation of TopBP1 with γ-H2AX in DNA damages (j–l).
sites of TopBP1 localization after DNA damage, we examined whether TopBP1 colocalizes with a phosphorylated histone variant γ-H2AX, which has been proved to sense and bind DNA strand breaks (23, 24) (Fig. 4E). The irradiation induced stable colocalization of TopBP1 and γ-H2AX in the nucleus (j and k), indicating the association of TopBP1 with the damaged DNA sites. In addition, the immunoreactivity of TopBP1 significantly increased in the damaged cells, supporting the irradiation-dependent stabilization of TopBP1 (d and j).

**Discussion**

The evidence for both ubiquitin-protein ligase activity of hHYD and TopBP1 as a substrate for hHYD-mediated ubiquitination may shed light on the molecular basis of cellular DNA damage response. TopBP1 was found to be usually ubiquitinated and degraded by the proteosomes in intact cells. X-irradiation seemed to abolish TopBP1 degradation and induced the stable complex formation of TopBP1 with other molecules in double strand DNA breaks. The finding that hHYD bound the region containing BRCT domains of TopBP1 suggests the possible association between hHYD and other BRCT-containing proteins. With DNA damage, BRCT-domain proteins are reported to be phosphorylated and are necessary for activation of many of the cell cycle checkpoint proteins (16, 18, 25). We observed that TopBP1 is also phosphorylated in irradiated cells. DNA damage stimulated the colocalization of TopBP1 with the sensing protein γ-H2AX into the DNA breaks, and TopBP1 is likely to contribute to the formation of a nuclear environment for the protection of DNA ends and for their repair. TopBP1 is associated with human DNA topoisoasemase II and appeared to be involved in DNA replication during the early S phase (26). Reduced expression of TopBP1 by antisense oligonucleotides decreased cell viability after DNA damage, suggesting the biological significance of TopBP1 (14).

Interestingly, the mutagen-sensitive-101 (mus101) gene of *D. melanogaster*, which links DNA repair, replication, and condensation of heterochromatin in mitosis, encoded a protein with seven BRCT domains highly analogous to the overall structure of TopBP1 (27). The mus101 mutants conferred larval hypersensitivity to DNA-damaging agents. Likewise, the yeast TopBP1-containing protein, Cut5, was demonstrated to interact with Chk1 kinase, a key molecule in DNA damage and cell cycle regulation (19). Thus, these BRCT-containing proteins may play similar roles in the DNA repair and cell cycle checkpoint.

**Hect-domain proteins participate in important physiological pathways, and the HECT domain is able to interact with E2 enzymes and assemble the ubiquitin chains. Meanwhile, the N-terminal extended region of the protein may mediate selective interactions with substrate proteins and factors involved in subcellular localization.** The evidence that TopBP1 is ubiquitinated by hHYD in vitro was clearly indicated in Fig. 2. Using similar assays shown in Fig. 3, however, we failed to directly analyze ubiquitinated forms of TopBP1 in the hHYD immunoprecipitates from the cells expressing wild- or active cysteine mutant-type hHYD together with TopBP1 and HA-tagged ubiquitin because repeated experiments unfortunately resulted in fluctuating levels of these proteins (data not shown). This might suggest the delicate balance of E3 enzyme and its substrate in the cell. Until now, human E6AP ubiquitinates tumor suppressor protein p53 in the presence of E6 oncoprotein from *Papillomavirus* (9–10) and HRH23A, one of the human homologues of the yeast DNA repair protein Rad23, as the E6-independent target (28). Interestingly, E6AP was found as the gene mutated in several cases of Angelman syndrome, a genetic neurobehavioral disorder that includes severe mental retardation, microcephaly, ataxic gait with jerky arm movements, hyperactivity and inappropriate laughter, and seizures (29, 30). Furthermore, NEDD4 is a ubiquitin ligase for the amiloride-sensitive epithelial sodium channel (22). The C terminus of each epithelial sodium channel subunit contains a PPXY motif, which is required for interaction with the WW domains of the NEDD4. Disruption of this interaction, as in Liddle’s syndrome, where mutations alter the PY motif of the subunit, results in increased epithelial sodium channel activity, leading to the heritable hypertension. We demonstrated that ubiquitination of TopBP1 by hHYD exemplifies ubiquitin conjugation linked to the DNA damage response. hHYD is the human homologue of the regulator of cell proliferation and the putative tumor suppressor hyperplastic discs in *D. melanogaster*. The alterations in the hyperplastic discs gene caused imaginal disc overgrowth and various developmental abnormalities (11, 12). Very recently, TopBP1 was reported to implicate in DNA replication and checkpoint control (31). In S phase, TopBP1 colocalizes with BRCA1 to foci that are not replicated DNA sites, meanwhile inhibition of DNA synthesis causes relocation of both TopBP1 and BRCA1 to replication forks. Thus DNA damage induces formation of distinct TopBP1 foci in S phase. TopBP1 also interacts with checkpoint protein hRad9. At present, the precise roles of TopBP1 in DNA damage and cell cycle control is not clear. Nevertheless, our data suggested the possible importance of hHYD-mediated ubiquitination of TopBP1 for cell regulation and DNA damage response.

**Acknowledgments**—We thank the Kazusa DNA Research Institute for the cDNA clone (GenBank accession number D87448) encoding TopBP1, Hoffmann-La Roche for providing the E2 expression vectors, and Y. Fukushima for secretarial assistance.

**References**

1. Hochstrasser, M. (1996) *Annu. Rev. Genet.* 30, 405–439
2. Cox, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* 65, 801–847
3. Jenuenn, H. P. (1995) *Eur. J. Biochem.* 231, 1–30
4. Ciechanover, A. (1994) *Cell* 78, 19–21
5. Santsch, S. (1992) *Annu. Rev. Genet.* 26, 179–207
6. Hershko, A., and Ciechanover, A. (1992) *Annu. Rev. Biochem.* 61, 761–807
7. Jackson, P. K., Eldridge, A. G., Freed, E., Furstenbuhl, L., Hsu, J. Y., Kaiser, B. K., and Reimann, J. D. R. (2000) *Trends Cell Biol.* 10, 429–439
8. Huhrebrg, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 2563–2567
9. Huhrebrg, J. M., Scheffner, M., and Howley, P. M. (1993) *Mol. Cell. Biol.* 13, 775–784
10. Scheffner, M., Huhrebrg, J. M., Vierstra, R. D., and Howley, P. M. (1993) *Cell* 75, 485–505
11. Mansfield, E., Herspereger, E., Bigges, J., and Sheffner, A. (1994) *Dev. Biol.* 165, 507–526
12. Martin, P., Martin, A., and Sheffner, A. (1997) *Dev. Biol.* 55, 213–232
13. Callaghion, M. J., Russell, A. J., Wiesllait, E., Sutherland, G. R., Sutherland, R. L., and Watts, C. K. W. (1998) * Oncogene* 17, 3479–3491
14. Yamane, K., and Tsuruo, T. (1999) * Oncogene* 18, 5194–5203
15. Yamane, K., Kawabata, M., and Tsuruo, T. (1997) * Eur. J. Biochem.* 250, 794–799
16. Anderson, L., Henderson, C., and Adachi, Y. (2001) * Mol. Cell. Biol.* 21, 1619–1729
17. Huyton, T., Bates, P. A., Zhang, X., Sternberg, M. J. R., and Freemont, P. S. (2000) *Mol. Biol. Rep.* 26, 319–332
18. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) *Science* 286, 1162–1166
19. Saka, Y., Esashi, F., Matsusaka, T., Mochida, S., and Yanagida, M. (1997) *Gene Dev.* 11, 3387–3400
20. Anan, T., Nagata, Y., Hoga, H., Honda, Y., Yabuki, N., Miamateo, C., Kawanu, A., Matsuda, I., Endo, F., Saya, H., and Nakao, M. (1998) *Genes Cells* 3, 751–763
21. Nagata, Y., Anan, T., Yoshida, T., Minkazumi, T., Saya, H., Fujiwara, T., Kato, C. S., Saya, H., and Nakao, M. (1999) * Oncogene* 18, 6057–6049
22. Harvey, K. F., and Kurnar, S. (1999) * Trends Cell Biol.* 9, 166–168
23. Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. W. (1977) *J. Cell Biol.* 104, 905–915
24. Rogakou, E. P., Plch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) *J. Biol. Chem.* 273, 5858–5868
25. Zhou, B-B., and Elledge, S. J. (2000) *Nature* 405, 433–439
26. Potter, T., Golde, W., Wedemeyer, N., and Kohnlein W. (2000) *Radiat. Res.* 154, 151–158
27. Yamamoto, R. R., Axton, J. M., Yamamoto, Y., Saunders, R. D., Glover, D. M., and Henderson, D. S. (2000) *Genetics* 151, 711–731
28. Kishino, T., Lalande, M., and Wastgaff, J. (1979) *Nat. Genet.* 15, 70–732
29. Makiem, N., Hiiukkuka, T., Tusa, J., Reini, K, Vaara, M., Huang, D., Knapich, H., Majola, L., Westering, T., Makela, T. P., Syrmou, J. E. (2001) *J. Biol. Chem.* 276, 30399–30406
Cooperation of HECT-domain Ubiquitin Ligase hHYD and DNA Topoisomerase II-binding Protein for DNA Damage Response
Yoshiomi Honda, Masahide Tojo, Kazuhito Matsuzaki, Tadashi Anan, Mitsuhiro Matsumoto, Masayuki Ando, Hideyuki Saya and Mitsuyoshi Nakao

J. Biol. Chem. 2002, 277:3599-3605.
doi: 10.1074/jbc.M104347200 originally published online November 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104347200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 10 of which can be accessed free at http://www.jbc.org/content/277/5/3599.full.html#ref-list-1