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A Novel UbcH10-Binding Protein Facilitates the Ubiquitinylation of Cyclin B *In Vitro*

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UbcH10 is known to act as a ubiquitin-conjugating enzyme (E2) for anaphase-promoting complex/cyclosome. Since some E2s support different ubiquitin ligases (E3), it is possible that UbcH10 interacts with other proteins. We cloned a novel protein named H10BH by using a yeast two-hybrid screening method with UbcH10 as bait. The carboxyl terminus of H10BH showed a weak homology to the HECT (homologous to E6-AP carboxyl terminus) domain, which is conserved in one of the families of E3. H10BH bound UbcH10, and the amino acid sequence between 235 and 257 was necessary for this binding. H10BH showed a self-ubiquitinylation activity in a HECT-like sequence-dependent manner. The carboxyl terminal half (amino acids 188–389) showed stronger activity than the full-length H10BH. Furthermore, the carboxyl terminal half of H10BH was able to bind cyclin B and ubiquitinylate cyclin B *in vitro*. These results suggest that H10BH functions as an E3 using UbcH10 for its E2.

Key words: cyclin B, HECT domain, UbcH10, ubiquitin conjugating enzyme, ubiquitin ligase.

Abbreviations: APC/C, anaphase promoting complex/cyclosome; HECT, homologous to E6-AP carboxyl terminus; Ubc, ubiquitin-conjugating enzyme.

In eukaryotes, selective protein degradation is largely mediated by the ubiquitin/proteasome system (*1*). This system plays an important role in many biological functions including cell-cycle progression, signal transduction, and immune responses. Proteasomes recognize the poly-ubiquitinylated chain attached to the protein to be degraded (*2–4*). Substrate recognition for poly-ubiquitinylation involves the ubiquitin conjugating system. The ubiquitin conjugating system requires three enzymes: E1, a ubiquitin activating enzyme, E2, a ubiquitin-conjugating enzyme (Ubc), and E3, a ubiquitin ligase (*5–8*). The ubiquitin ligase recognizes the target protein to be degraded. There are at least two types of ubiquitin ligases (*9*). One type has a RING-finger domain, and the other has a HECT (homologous to E6-AP carboxyl terminus) domain. RING-finger type ubiquitin ligase catalyzes the direct transfer of the ubiquitin moiety from E2 to the substrate, and shows a self-ubiquitinylation activity when its substrate is absent (*10*, *11*). On the other hand, HECT type ubiquitin ligase first accepts the ubiquitin moiety from E2 and then transfers it to the target protein (*9*, *11*, *12*). Recently, U-box proteins and PHD domain proteins, both of which have RING-like sequences, are suggested to be new families of ubiquitin ligases (*12–14*).

The anaphase-promoting complex/cyclosome (APC/C) is a multi-subunit ubiquitin ligase that targets mitotic regulators including securin and cyclin B for degradation to initiate anaphase and exit from mitosis (*15*). APC/C contains at least 11 subunits in mammals, and cullin-like protein APC2 and RING-finger protein APC11 are believed to be the core subunits of APC/C (*16–18*). The activity of APC/C is tightly regulated by its phosphorylation states and regulators such as Mad2 (*19*, *20*). Cdc20/Fizzy or Hct1/Fizzy-related associates APC/C and is thought to represent the substrate specificity (*20*, *21*). APC/C utilizes UbcH10 (human)/UbcX (frog)/E2-C (Clam) and UbcH5 (human)/Ubc4 (budding yeast) as its E2s (*16*, *17*, *22–24*). When dominant-negative UbcH10 or E2-C was introduced into dividing cells, the destruction of mitotic cyclins was inhibited, and cell accumulation of in mitosis before anaphase onset was observed (*23*). These results suggest that UbcH10/UbcX/E2-C is the main E2 involved in the progression of mitosis with APC/C.

As each E3 uses several E2s, the same E2s support different types of E3s. UbcH5 acts as an E2 for APC/C (*25*) and also Mdm2 or E6-AP that ubiquitinylate p53 (*26*, *27*). UbcH7 can bind and support c-Cbl to ubiquitinylate growth factor receptors (*28*) and also E6-AP (*29*). Thus, it is possible that UbcH10 interacts with different E3s or proteins, although UbcH10 has been reported to bind APC11 (*18*). In order to identify proteins that interact with UbcH10, we utilized a yeast two-hybrid screening method with UbcH10 as bait. We then were able to identify a novel protein with a HECT-like sequence at the carboxyl terminus, which had a self-ubiquitinylation activity and ubiquitinylated cyclin B *in vitro*.

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EXPERIMENTAL PROCEDURES

Cloning of UbcH10-Interacting Protein—The cDNA for the coding region of UbcH10 was fused to pGBT9 (BD Biosciences Clontech, San Jose, CA) plasmid, which contains a GAL4 DNA-binding domain. Screening in the yeast two-hybrid system was performed according to the manufacturer's protocol using a mouse T-cell lymphoma cDNA library in pACT plasmid containing a GAL4-activating domain (BD Biosciences Clontech, San Jose, CA). DNA sequencing was performed by the dye-termination procedure using an ALF Auto Sequencer (Amersham Biosciences, Piscataway, NJ) or ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

To obtain the human homologue of mouse clone No. 10, 5′RACE reactions were performed with Marathon-Ready HeLa cDNA as a template according to the manufacturer's protocol. The 3′ end primers were designed from the sequence of human EST clone (GenBank accession No. AA136213), which showed the highest homology to clone No. 10 by BLAST search: first primer, CTGGATTCCACACAGGACAGATGG; nested primer, GCGCGGCCGCCTTCCAGGTGGTGCTGACACTGC. The amplified fragment was sequenced and used as a probe for screening of HeLa cDNA λZAPII library to obtain full-length cDNA. Further 5′RACE reactions were also performed to identify the 5′ end.

Recombinant Proteins Expression and Preparation—UbcH10 and UbcH5c were expressed in *Escherichia coli* strain BL21 (DE3, pLysS) and purified by ammonium sulfate precipitation followed by Mono Q column (Amersham Biosciences, Piscataway, NJ) chromatography. GST- or His-tagged H10BH and GST- or no-tagged mouse cyclin B1 were expressed in Sf9 cells by using a baculovirus protein expression system according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ). Truncated mutants of H10BH and cyclin B were generated by PCR reactions of full-length cDNA. Substitute mutants in the HECT-like domain (C358A and T357A, C358S) of H10BH and destruction-box (R42A, L45A) of cyclin B were generated by a PCR-based *in vitro* mutation method using specific mutation primers.

To purify the proteins, Sf9 cells expressing recombinant proteins were suspended in buffer A (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, 0.1 % NP-40), and then disrupted by brief sonication. After centrifugation at 14,000 × *g* for 10 min, the precipitate was extracted with buffer A containing 0.4 M NaCl for 15 min at 4°C. The first supernatant and the extract were mixed and centrifuged at 14,000 × *g* for 10 min. The resulting supernatant was incubated with glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) for 30 min at 4°C, and the beads were washed four times with buffer A containing 0.4 M NaCl.

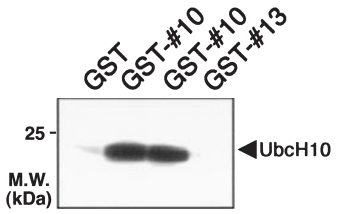


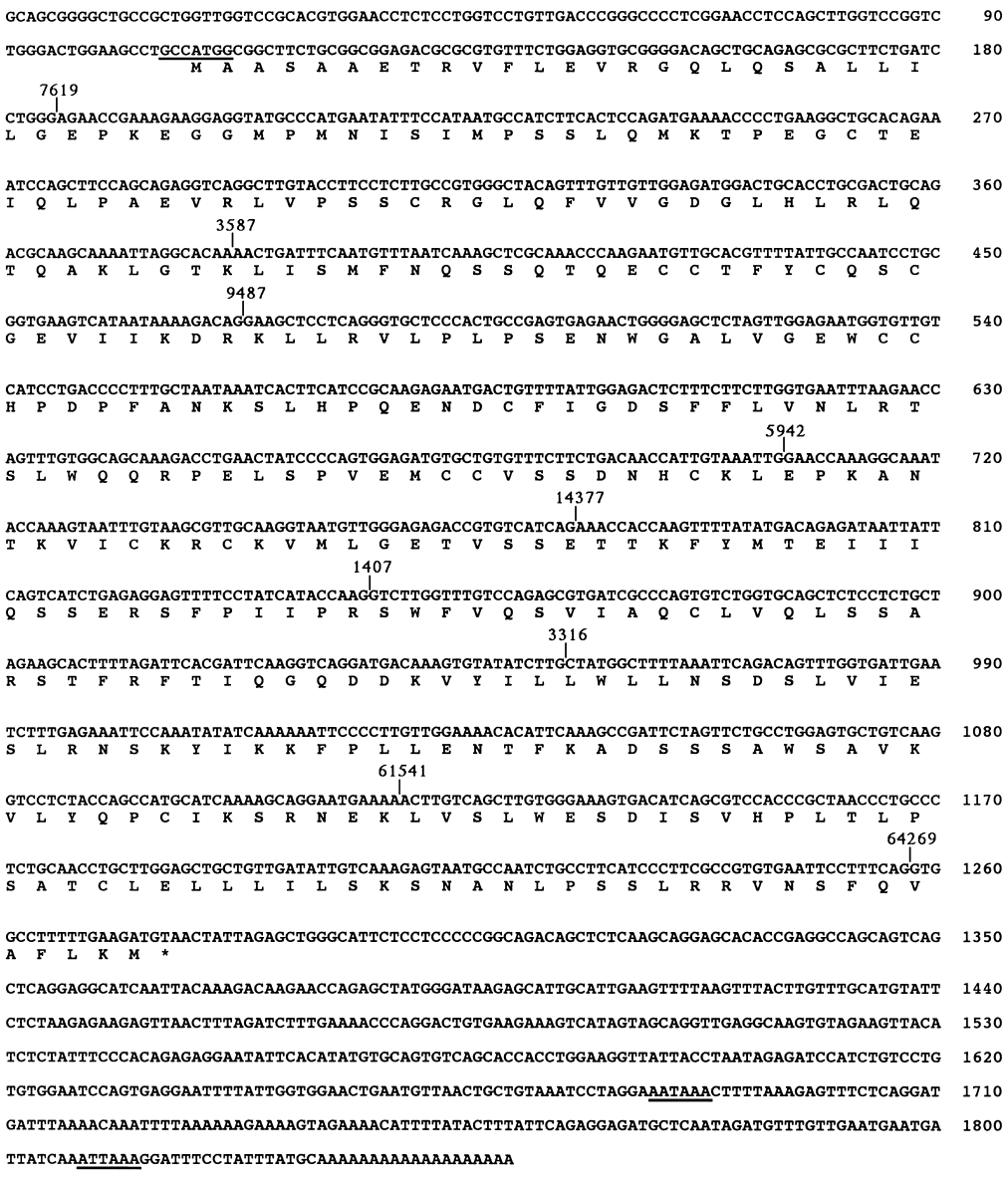
Fig. 1. **UbcH10 interacts with mouse clone No. 10.** GST or GST-fusion protein of mouse clone No. 10, a positive clone in a yeast two-hybrid screening method, was expressed in Sf9 cells and captured on glutathione Sepharose 4B. Beads were incubated with biotinylated UbcH10 for 30 min at 25°C, and bound proteins were eluted and separated by SDS-PAGE. Biotinylated UbcH10 was detected by use of avidin-peroxidase and ECL. GST-fusion protein of clone No. 13, which is a false positive clone obtained during the yeast two-hybrid screening method, was also examined.

Binding Assay—The GST-H10BH captured on beads was incubated with biotinylated UbcH10 at 25°C for 30 min, and then the beads were washed four times with buffer A containing 0.4 M NaCl. The bound proteins were eluted with an SDS sample buffer, subjected to SDS-PAGE, transferred to PVDF membrane, and detected by ECL method (Amersham Biosciences, Piscataway, NJ) using ExtrAvidin-peroxidase (Sigma Chemical, St. Louis, MO) as a probe. For the binding assay between H10BH and cyclin B, the supernatants of Sf9 cells expressing GST- or His-tagged H10BH and no- or GST-tagged cyclin B were mixed and incubated for 30 min at 25°C, followed by incubation with glutathione-Sepharose 4B for 30 min at 4°C. After washing the beads as described above, the bound proteins were detected by Western blot analysis using an anti-His6 antibody (Qiagen K.K., Tokyo) or an anti-cyclin B antibody and a peroxidase-conjugated secondary antibody, respectively. The membranes were stained with PhastGel BlueR (Amersham Biosciences, Piscataway, NJ) to compare the protein amounts used in the assay.

Ubiquitinylation Assay—GST-H10BH captured on glutathione-Sepharose 4B was incubated with purified E1 (2 μg), purified UbcH10 (1 μg), and biotinylated ubiquitin (10 μg) in total volume of 50 μl of ubiquitinylation buffer (50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM ATP) for 30 min at 25°C. After washing the beads, proteins bound to the beads were subjected to SDS-PAGE and transferred to a PVDF membrane. Proteins conjugated with biotinylated ubiquitin were detected by the method mentioned above. To compare the specificity of E2s, purified UbcH5c, or the lysate of *E. coli* expressing human Cdc34 or UbcH7 was substituted for

Fig. 2. **Nucleotide and predicted amino acid sequences of human H10BH.** (A) Nucleotide and predicted amino acid sequences of human homologue of mouse clone No. 10. The conserved Kozak's sequence and two poly (A) signals are underlined. Intron positions and nucleotide lengths are indicated on the nucleotide sequence. (B) Alignment of human HECT proteins and H10BH relative to the 39 carboxyl-terminal amino acids of E6-AP. All known proteins except UREB1 have been reported to be ubiquitin ligases. GenBank accession numbers are follows: E6-AP, L07557; H10BH, AB126062; HYD, U95000; Itch, AB056663; NEDD4, NM_006154; SMURF2, AF301463; WWP1, AY043361; KIAA0010, D13635; UREB1, AF071605. Conserved amino acids in more than five proteins are indicated in bold type, and identical amino acids in all proteins are indicated by * under the alignment. Conserved active cysteine residue is also indicated by #. The percent identity of each protein to E6-AP over these amino acids is shown to the right.

A



B

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                                                    % identity to E6-AP
                    #
E6-AP     :  LPTSHTCFNVLLLPEYSSKEKLKERLLKAI-TYAKGF-GML
H10BH     :  LP-SATC-LELLLILSKSNANLPSSLRRVN-SFQVAF-LKM        31
HYD       :  LPTANTCISRLYVPLYSSKQILKQKLLLAI--KTKNF-GFV        51
Itch      :  LPRSHTCFNRLDLPPYKSYEQLKEKLLFAI-EETEGF-GQEUTR    57
NEDD4     :  LPRAHTCFNRLDLPPYESFEELWDKLQMAI-ENTQGFDGVD       50
SMURF2    :  LPKAHTCFNRIDIPPYESYEKLYEKLLTAI-EETCGF-AVE       51
WWP1      :  LPRSHTCFNRLDLPPYKSYEQLKEKLLFAI-EETEGF-GQE       62
KIAA0010  :  LPTASTCMNLLKLPEFYDETLLRSKLLYAI-ECAAGF-ELS       46
UREB1     :  LPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGF-GLA       55
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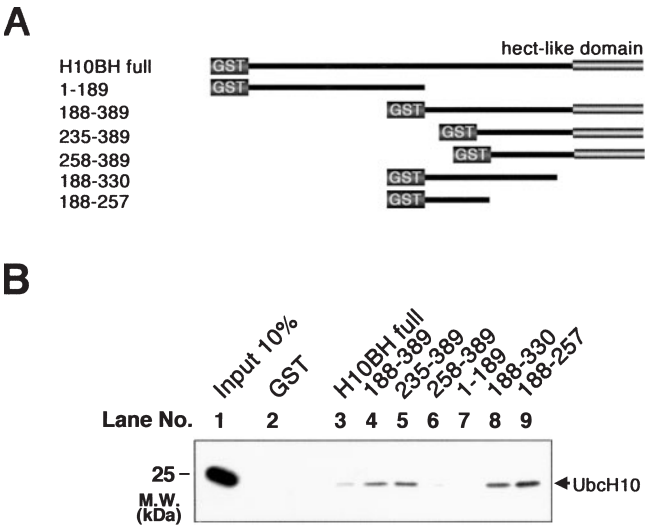

Fig. 3. **UbcH10 binds to the specific portion of H10BH.** (A) Schematic representation of GST-H10BH proteins. GST proteins contain the H10BH amino acids indicated to the left. The shaded region of each represents the HECT-like sequence. (B) The same amount of GST or each of the GST-H10BH proteins captured onto glutathione Sepharose 4B was incubated with biotinylated UbcH10 at 25°C for 30 min. UbcH10 bound to GST proteins was detected as described in "MATERIALS AND METHODS." The right lane represents 10% of the input UbcH10 used in each assay.

UbcH10 at the concentration that showed the same E2 activity. For the ubiquitinylation assays of cyclin B by H10BH, lysate of Sf9 cells expressing GST-H10BH (188–389) or mutants was first mixed with lysate of Sf9 cells expressing GST-cyclin B or GST-fusion proteins which were captured on glutathione-Sepharose 4B. After washing, the beads were subjected to the ubiquitinylation assay as described above.

RESULTS AND DISCUSSION

Cloning of UbcH10 Binding Protein—The full-length coding region of human UbcH10 was used to generate a fusion protein with a GAL4 DNA-binding domain. Using this plasmid, a mouse T-cell lymphoma cDNA library was screened by a yeast two-hybrid method. Two positive clones (Nos. 8 and 10) were obtained, and DNA sequencing revealed that these clones were identical and contained an open reading frame coding 201 amino acids with a C-terminal stop codon but lacked an N-terminal in-frame stop codon. As a result of our BLAST search, we found that the protein had no homology with known proteins except EST clones. To confirm the direct binding of this protein to UbcH10, we carried out an *in vitro* binding assay. As shown in Fig. 1, GST-clone No. 10 bound to UbcH10, but neither GST nor GST fusion protein of clone No. 13, which is a false positive clone obtained during a yeast two-hybrid screening method, bound to UbcH10. We confirmed that clone No. 10 is a novel UbcH10-binding protein.

To obtain the full-length human homologue of clone No. 10, we carried out 5′ RACE method and cDNA library screening. For the 5′ RACE reaction, the primers were designed from a human EST clone (GenBank accession No. AA136213) which showed a high homology to clone No. 10. The amplified DNA fragment from Marathon-ready HeLa cDNA was used as a probe for the screening of the HeLa cell cDNA library. The DNA sequence and deduced amino acid sequence of the longest clone obtained are shown in Fig. 2A. This clone contains an open reading frame coding 427 amino acids and a poly (A) tail preceded by two poly (A) signals. There is no stop codon preceding the first methionine. However, we could not get any longer 5′ end fragment by further 5′ RACE reactions using several sets of primers, and the Kozak's sequence was conserved around the first ATG. We assigned the initiating methionine to the first ATG of the cDNA, and this gene encoded the protein of 389 residues with a calculated molecular mass of 43.7 kDa. By Northern analysis using total RNA isolated from HeLa cells, we identified a transcript for this gene of approximately 1.8 kb (data not shown). This size fits to the cDNA and indicates that the isolated cDNA contains the complete open reading frame. The BLAST search of the human genome revealed that this gene consists of 10 exons and expands to approximately 173 kbp on the long arm of chromosome 6.

A comparison of the deduced amino acid sequence revealed that 176 amino acids from the carboxyl terminus showed 17% identity and 63% homology to the HECT domain of E6-AP, which is a consensus sequence of one of the ubiquitin ligase families. The highest identity (31%) was observed at the C-terminus 37 amino acids (Fig. 2B). Compared to the other HECT domain proteins, the homology of this protein to E6-AP is low, but the essential cysteine residue for E3 activity is conserved. We named the gene H10BH (UbcH10 binding protein with a HECT-like domain).

The full-length human H10BH fused with GST also bound to UbcH10 (Fig. 3B) as did mouse clone No. 10. In order to map the H10BH sequence that directed this interaction, we synthesized various portions of H10BH as GST fusion proteins (Fig. 3A). The carboxyl-terminal portion (amino acids 188 to 389) bound to UbcH10 stronger than the full-length protein, but the amino-terminal portion (amino acids 1 to 189) did not. Additional truncations of the carboxyl-terminal portion (amino acids 188 to 389) from the amino-terminus or the carboxyl-terminus localized the UbcH10-binding region within the 23–amino-acid region between amino acids 235 and 257 (Fig. 3B). Consequently, this region was found to be located outside the HECT-like domain (amino acids 353 to 389).

Self-Ubiquitinylation Activity of H10BH—The HECT-type ubiquitin ligase accepts ubiquitin from E2 in the form of a thioester and then directly transfers ubiquitin to the target substrate (*18*, *30*). Since H10BH has a HECT-like domain with a conserved cysteine residue, it may act as an E3 ubiquitin ligase. After the incubation of GST-H10BH with E1, UbcH10, biotinylated ubiquitin, and ATP, the ubiquitinylated proteins were separated by SDS-PAGE, transferred to a membrane and detected by avidin peroxidase. A ubiquitinylated band was detected at the position of the mono-ubiquitinylated form of GST-H10BH (Fig. 4A). This conjugation was resistant to dithiothreitol treatment as reported in some HECT proteins (*30*). The fact that pretreatment of GST-H10BH with *N*-ethylmaleimide inhibited ubiquitinylation suggests that

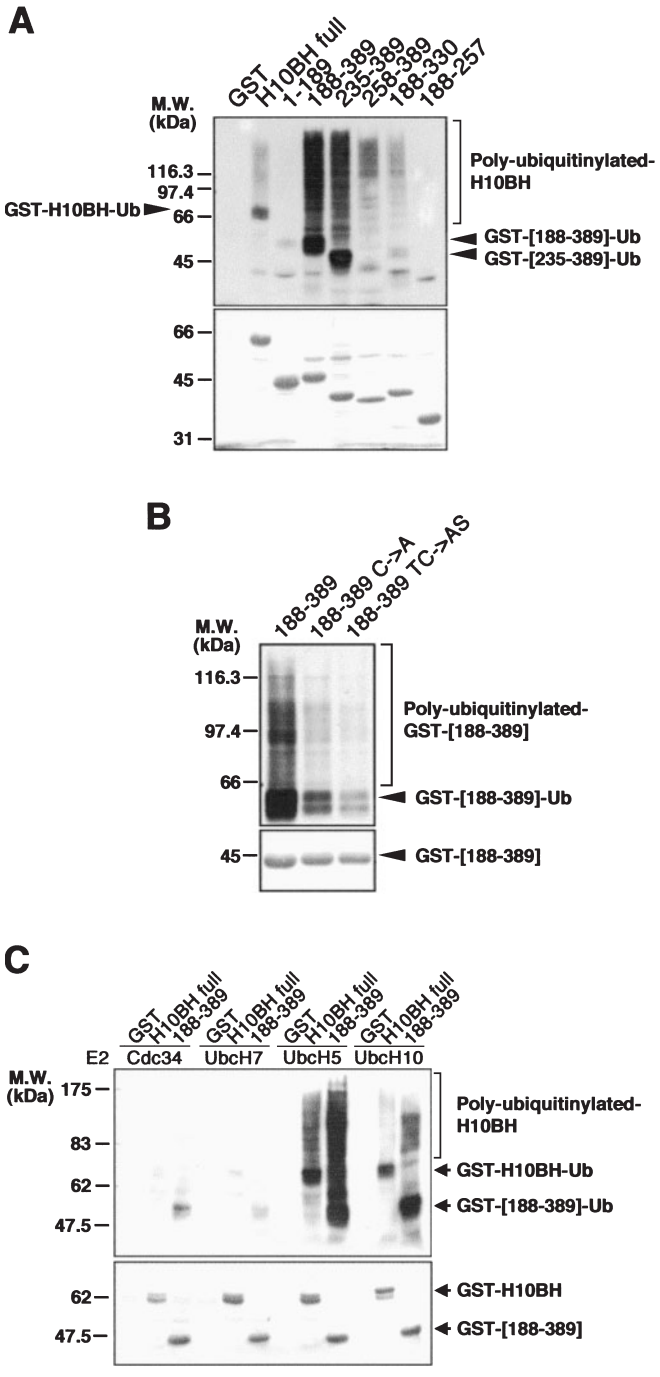

Fig. 4. **H10BH possesses self-ubiquitinylation activity.** (A) Each GST-H10BH protein shown in Fig. 3 (A) was captured on glutathione Sepharose 4B, and ubiquitinylation assay was performed in the presence of E1, UbcH10 and biotinylated ubiquitin as described in "MATERIALS AND METHODS" (upper panel). Protein staining of the membrane was shown to compare the protein amount used (lower panel). (B) The effects of substitution mutations (C358A or T357A, C358S) in HECT-like domain of GST-H10BH (188–389) on the self-ubiquitinylation activities were examined (upper panel). Protein staining of the membrane is shown (lower panel). (C) The specificity of E2 for the self-ubiquitinylation activity of GST-H10BH proteins was examined. Purified UbcH10 or UbcH5c, or the lysate of *E. coli* expressing human Cdc34 or UbcH7 was added to the self-ubiquitinylation reaction of GST-H10BH or GST-H10BH (188–389) as indicated in the upper panel. Ubiquitinylated proteins are shown in the lower panel.

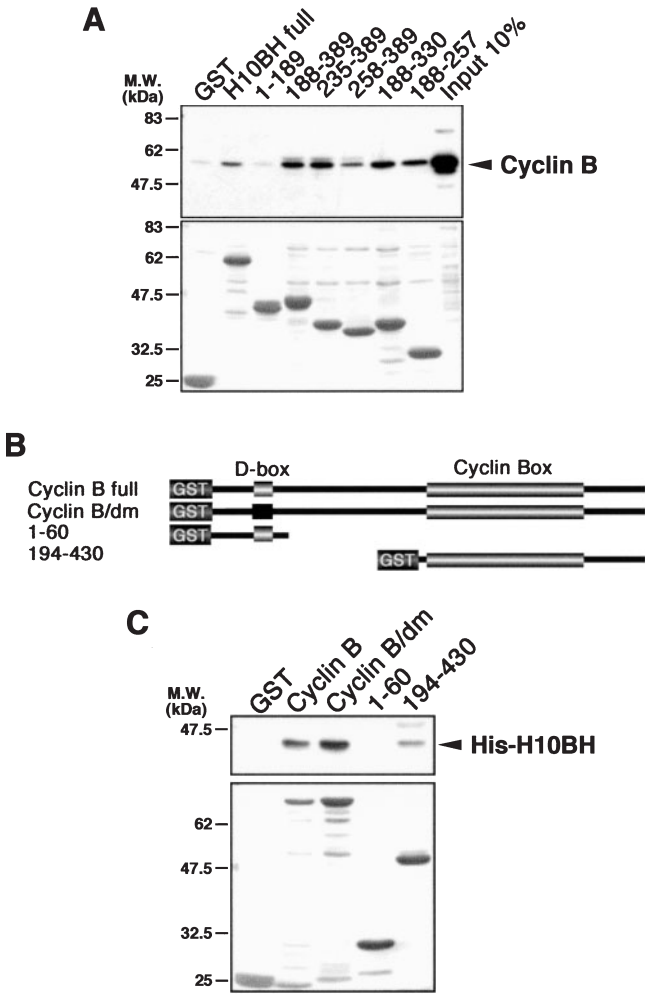

Fig. 5. **H10BH interacts with cyclin B.** (A) Each GST-H10BH protein shown in Fig. 3 (A) was captured on glutathione Sepharose 4B and incubated with the lysate of Sf9 cells expressing full-length cyclin B. Cyclin B binding to GST-H10BH proteins was detected by Western blot analysis with anti–cyclin B antibody (upper panel). The right lane represents 10% of the input cyclin B. Protein staining of the membrane is shown in the lower panel. (B) Schematic representation of GST-fused cyclin B proteins. The right short box represents the destruction box, and the left shaded box the cyclin box in cyclin B. Cyclin B/dm contains mutations (R42A, L45A) in the destruction box. (C) The lysate of Sf9 cells expressing GST-cyclin B proteins was mixed with the lysate of Sf9 cells expressing His-H10BH, and incubated for 30 min at 25°C, followed by incubation with glutathione-Sepharose 4B for 30 min at 4°C. His-H10BH bound to GST-cyclin B was detected by Western blot analysis using Anti-His6 antibodies (upper panel). The right lane represents the 1% input of His-H10BH. Protein staining of the membrane is shown (lower panel).

a cysteine(s) in H10BH is essential for this reaction (data not shown). To determine the region essential for the self-ubiquitinylation, truncated forms of GST-H10BH, the same as in Fig. 3A, were examined (Fig. 4A). The carboxyl terminal portion (amino acids 188–389) was strongly mono- and poly- or multi-ubiquitinylated, but the amino terminal portion (amino acids 1–189), which could not bind UbcH10, was not ubiquitinylated. This result suggests that the amino terminus region has an inhibitory effect on self-ubiquitinylation of H10BH. As

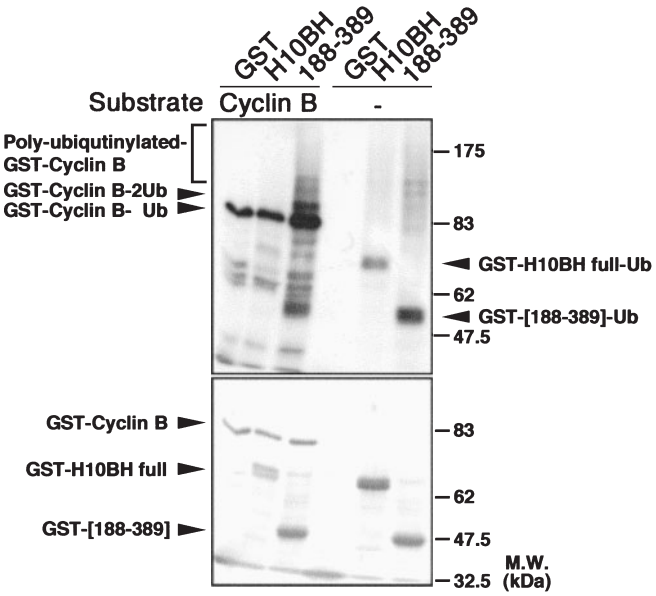

Fig. 6. **Cyclin B is ubiquitinylated by H10BH.** The lysate of Sf9 cells expressing GST-H10BH or GST-H10BH (188–389) was incubated with the lysate of Sf9 cells expressing GST-cyclin B as indicated in the upper panel. GST-fusion proteins were purified by glutathione Sepharose 4B and subjected to ubiquitinylation reaction with E1, UbcH10, and biotinylated ubiquitin. Ubiquitinylated proteins were detected as described in "MATERIALS AND METHODS" (middle panel). The lower panel shows the protein staining of the membrane.

expected, the UbcH10 binding region (amino acids 235–257) was essential for the self-ubiquitinylation. Furthermore, deletion of the carboxyl terminal 59 amino acids diminished the self-ubiquitinylation activity (Fig. 4A), suggesting the importance of the HECT-like domain. To confirm the involvement of the HECT-like domain in self-ubiquitinylation, mutants were created in which alanine was substituted for the conserved cysteine residue at 358 or alanine and threonine were substituted for threonine 357 and cysteine 358 of the carboxyl terminal half portion (amino acids 188–389). The self-ubiquitinylation activities of these mutants were diminished (Fig. 4B). These results suggest that the HECT-like domain, especially the conserved cysteine residue, is essential for the self-ubiquitinylation activity of H10BH.

To determine the specificity of E2, self-ubiquitinylation activities of GST-H10BH and GST-H10BH (188–389) were examined by using several E2s. Both Cdc34, which is specific for RING-finger type E3 SCF (*31*), and UbcH7, which supports E6-AP (*29*), failed to support the self-ubiquitinylation of H10BHs (Fig. 4C). On the other hand, UbcH5 showed a high E2 activity towards H10BH. UbcH5 is known to act as an E2 for many E3s including APC/C (*25*), suggesting that H10BH utilizes both of UbcH10 and UbcH5 as its E2 *in vitro*. The preferential E2 for UbcH10 *in vivo* should be further elucidated.

H10BH Binds and Ubiquitinylates Cyclin B—Since UbcH10 is known to act as an E2 for APC/C and supports ubiquitinylation of cyclin B and other mitotic proteins (*32*, *33*), it is possible that H10BH also binds and ubiquitinylates cyclin B. We first examined the binding of H10BH and cyclin B. Full-length H10BH bound to cyclin B, and the carboxyl terminal half showed a stronger binding (Fig. 5A). The binding region to cyclin B was mapped mainly at amino acids 235 to 257, which is the same region that binds to UbcH10 (Fig. 5A). Cyclin B has two well-known domains: the destruction box in the amino terminal region, which is essential for the ubiquitinylation by APC/C; and the cyclin box in the carboxyl terminal region, which is conserved among cyclins. His-tagged H10BH bound to GST-fused cyclin B with a mutation in the destruction box as well as to the wild type (Fig. 5B). Also, we found that H10BH could not bind to the amino terminal 60 amino acids of cyclin B, the region that contained the destruction box. On the other hand, H10BH was found to bind to the carboxyl terminal half portion. These results indicate that H10BH recognizes the carboxyl terminal region of cyclin B, which contains the cyclin box, but not the destruction box.

To confirm whether cyclin B is ubiquitinylated by H10BH, we carried out an *in vitro* ubiquitinylation assay with GST-H10BH and GST-cyclin B bound to GSH-Sepahrose (Fig. 6). Mono-ubiquitinylation of cyclin B was detected only in the presence of E1 and UbcH10. This phenomenon is not surprising, since it has been reported that Ubc4 itself ubiquitinylates cyclin B *in vitro* (*30*). The carboxyl terminal portion of H10BH (amino acids 188–389), which possesses a strong self-ubiquitinylation activity, greatly stimulated the mono-ubiquitinylation of cyclin B, and furthermore, both di-ubiquitinylation and some poly- or multi-ubiquitinylation were observed. No additional effect of the full-length H10BH may be due to the weak binding affinities to UbcH10 and cyclin B, and weak self-ubiquitinylation activity. We suggest that the amino terminal portion functions as a regulatory domain and some modifications or other factors are required to exert full ubiquitinylation activities.

In this report, a novel protein, H10BH, was isolated as a binding protein to UbcH10 using a yeast two-hybrid screening method. H10BH contains a sequence weakly homologous to the HECT domain, which is reported to be contained in several ubiquitin ligases. H10BH exhibits self-ubiquitinylation activities. Furthermore, H10BH, especially its carboxyl terminus portion, not only binds but was found to also ubiquitinylate cyclin B *in vitro*. APC11, a RING-finger protein, is reported to be the core catalytic subunit of APC/C and to be sufficient to ubiquitinylate cyclin B in a destruction box-independent manner (*15*, *18*, *34*). Cdc20/fizzy and Cdh1/fizzy-related are believed to recognize the substrate specificity including the presence of the destruction box and support the ubiquitin ligase activity of APC/C (*20*, *21*). H10BH does not interact with the destruction box of cyclin B and this suggests a requirement of other factors for the proper ubiquitinylation of cyclin B. Another possibility is that H10BH indeed ubiquitinylates cyclin B in a destruction box-independent manner. The weak activities of self- and cyclin B-ubiquitinylation of full-length H10BH compared to the carboxyl terminal portion also suggest that the regulatory mechanism for the activation of H10BH is required. Preliminary experiments showed that the ubiquitinylation activity in the lysate of HeLa cells synchronized at late mitosis was greatly enhanced by the addition of H10BH. This raises two possibilities. One is that the activator(s) for H10BH is present in the lysate of HeLa cells.

The other possibility is that H10BH facilitates the ubiquitin ligase activity of APC/C. Ufd2 protein has been reported as such a factor which supports the E3 activity to generate a poly-ubiquitin chain to a substrate, and has been assigned as a ubiquitin chain assembly factor (E4) (*14*).

This is the first report of a UbcH10-interacting protein other than APC/C. Since the same E2 supports several ubiquitin ligases, UbcH10 may act as an E2 to another E3 with another substrate specificity. Thus, H10BH may ubiquitinylate yet unknown substrates more efficiently. The function of H10BH in cells should be further elucidated.

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