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Degradation of Human Exonuclease 1b upon DNA Synthesis Inhibition

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Abstract

In response to DNA damage, signaling pathways are triggered that either block the cell division cycle at defined transitions (G1-S and G2-M) or slow down progression through the S phase. Nucleases play important roles in DNA synthesis, recombination, repair, and apoptosis. In this study, we have examined the regulation of human exonuclease 1 (hEXO1b). The endogenous hEXO1b protein was only detected upon enrichment by immunoprecipitation. We found that hEXO1b was constantly expressed throughout the cell cycle. However, treatment of cells with agents that cause arrest of DNA replication led to rapid degradation of hEXO1b. This effect was fully reversed upon removal of the block. Analysis of synchronized cells showed that degradation of hEXO1b during the S phase was strictly dependent on DNA synthesis inhibition. DNA damage caused by UV-C radiation, ionizing radiation, cisplatin, or the alkylating agent N-methyl-N-nitro-N-nitrosoguanidine did not affect hEXO1b stability. We show that hEXO1b was phosphorylated in response to inhibition of DNA synthesis and that phosphorylation coincided with rapid protein degradation through ubiquitin-proteasome pathways. Our data support the evidence that control of exonuclease 1 activity may be critical for the maintenance of stalled replication forks. (Cancer Res 2005; 65(9): 3604-9)

Introduction

Nucleases play important roles in DNA synthesis, recombination, repair, and apoptosis. Exonuclease-1 (Exo1) was originally identified in Schizosaccharomyces pombe (1) as a member of the Rad2 family of structure-specific nucleases (2). Saccharomyces cerevisiae Exo1 was found to be highly transcribed in meiosis and the encoded protein was shown to participate in processing double-strand break ends as well as in meiotic crossover (3). The sequence of the human homologue of S. cerevisiae Exo1 gene revealed only 27% identity at the amino acid level (4). Nonetheless, human exonuclease 1 (hEXO1) was shown to be functionally similar to the yeast protein by its ability to complement S. cerevisiae Exo1p (5). Two isoforms of hEXO1 (hEXO1a and hEXO1b) were described to arise from alternative splicing, although no functional differences between the two products have yet been observed (6).

Exonuclease 1 catalyzes the removal of mononucleotides from the 5’ end of the DNA duplex, showing a strong preference for blunt-ended, 5’-recessed termini and DNA nicks. It can also exonucleolytically degrade ssDNA although less efficiently than double-stranded DNA. Moreover, hEXO1b displays a 5’-ssDNA-flap-specific endonuclease activity (2, 7).

Mismatch repair is a postreplication DNA repair mechanism that is implicated in reducing the rate of somatic microsatellite polymorphism and is disabled in a number of human tumors (8). S. pombe Exo1p was shown to play a key role in mismatch correction and mutation avoidance (9). Physical and genetic interaction between yeast Exo1p and the proteins Msh2p (10) and Mlh1p (11) provided direct evidence for the functional and a structural role of Exo1p in mismatch repair (12). Interaction with mismatch repair proteins was also observed in human cells where hEXO1 formed a complex with hMLH1/hPMS2 (13). Studies conducted with reconstituted systems pointed to hEXO1 as the most likely candidate for the excision step during mismatch repair in mammals (4, 14, 15). In addition to its role in the mismatch repair system, S. cerevisiae Exo1p was shown to be involved in mitotic recombination (16) and end resection at unprotected telomeres (17). A role for hEXO1 in resolving DNA intermediates formed during recombination was suggested by the finding that the protein physically interacted with the Werner syndrome helicase (18). Colocalization of ectopically expressed hEXO1 with proliferating cell nuclear antigen (PCNA) further pointed to a potential role for hEXO1 in controlling events at sites of replication (19). The involvement of hEXO1 in the control of key metabolic processes is emphasized by the phenotype of Exo1−/− mice that displayed reduced survival, sterility, and increased susceptibility to the development of lymphomas (20). Analysis of Exo1−/− mouse cells revealed specific defects in mismatch repair leading to elevated microsatellite instability, increase in mutation rate at the Hprt locus, abnormal spindle structures in metaphase cells (20), altered somatic hypermutation, and reduced class switch recombination (21). In this study, we provide evidence that hEXO1b is destabilized in response to replication fork arrest by hydroxyurea and aphidicolin but not upon generation of adducts in DNA or double-strand breaks. We show that rapid hEXO1b protein degradation occurs via an ubiquitin-mediated proteasome pathway and is facilitated by phosphorylation.

Materials and Methods

Expression vectors, chemicals, and antibodies. The coding region of hEXO1b (codons 1-846) was amplified by PCR using human EXO1b cDNA as template and the primers 5’-GGGAATTCCATATGCGGATACAGG-GATTGCTA-3’ (forward) and 5’-AAGGCCGCTCTCTCCGACCATGGA-TATGCTCTTGGAAACCG-3’ (reverse). The PCR product was inserted between the NdeI and SapI sites of the pTXB1 plasmid (NEB) to construct a translational fusion between hEXO1b and a self-cleaving affinity tag that is composed of an Mse intein fragment and the chitin-binding domain. A plasmid encoding a fusion between the NH2-terminal region of hEXO1b (codons 1-391) and Mxe–chitin-binding domain was constructed in the same way as indicated above using the primers 5’-GGGAATTCCATATGGCGGATACAGG-GATTGCTA-3’ (forward) and 5’-AAGGCCGCTCTCTCCGACCATGGA-TATGCTCTTGGAAACCG-3’ (reverse). An additional methionine codon

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was placed between hEXO1b and the affinity tag according to the manufacturer's instructions. Human EXO1a was generated as indicated above using the primers 5'-GGAGGATCCCATATGGGATACAGGGTGTCA-3' (forward) and 5'-AAGGGCCGCTTCTCAGGAAATTTTTAATCACCAGTGTTC-3' (reverse). Constructs were verified by sequencing. Recombinant hEXO1b, hEXO1b 1-391, and hEXO1a proteins were produced in the Escherichia coli BL21-CodonPlus-(DE3)-RIL cells (Stratagene, La Jolla, CA) harboring the plasmids described above and purified using chitin beads as previously described (22). Expressed proteins were further purified on a 5-mL HighTrap SP column (Amersham Pharmacia, Uppsala Sweden) equilibrated with a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% (v/v) glycerol, 0.1 mmol/L EDTA, and 1 mmol/L DTT. Proteins were eluted with a linear concentration gradient of NaCl (0.2-0.45 mol/L; 50 mL) and stored at −80°C.

Full-length human EXO1b cDNA was subcloned in the mammalian expression vector pcDNA3.1/His (Invitrogen, Carlsbad, CA) between the BamHI and EcoRV sites. A polyclonal antibody to hEXO1b 1-391 (F-15) was generated in rabbit (Clonestart, Brno, Czech Republic) and purified using a protein A-Sepharose column (Amersham Pharmacia) according to the manufacturer's instructions. A monoclonal antibody to full-length EXO1b (Ab-4, clone 266) was purchased from NeoMarkers (Fremont, CA). Antibodies to hChk1-pSer345 and hChk2-pThr68 were from Cell Signaling Technology (Beverly, MA). Antibodies to TFIH (sc-293) and hChk2 were from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Beverly, MA). Antibodies to TFIIH (sc-293) and hChk2 were from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Beverly, MA), respectively. Hydroxyurea, cisplatin, aphidicolin, and nocodazole were obtained from Sigma (St. Louis, MO) and dissolved in water or DMSO as specified by the manufacturer. MG132 and okadaic acid were purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. Ubiquitin was detected with monoclonal antibody (Ab-4, clone 266) purchased from NeoMarkers (Fremont, CA). Antibodies to hChk1-pSer345 and hChk2-pThr68 were from Cell Signaling Technology (Beverly, MA). Antibodies to hEXO1b 1-391 (F-15) were captured from 293 T cells with antibody F-15, the antibody was captured from 293 T cells with antibody F-15, and probed with monoclonal antibody Ab-4.

**Results**

**Characterization of antibodies to hEXO1b.** As prerequisite to exploring the effect of DNA damage on hEXO1, we set out to characterize the expression of endogenous hEXO1 protein. The polyclonal antibody F-15 could detect recombinant hEXO1b protein expressed in *E. coli* (Fig. 1A, lanes 1 and 2) and hEXO1b ectopically expressed in 293 T cells (Fig. 1A, lane 7) that were used as positive controls. However, neither monoclonal antibody Ab-4 (data not shown) nor our F-15 polyclonal antibody was able to detect endogenous hEXO1 in HEK 293T (Fig. 1A, lanes 4 and 5). hELa, U2-OS cells, or human fibroblasts (data not shown). Endogenous hEXO1b was detected by Western blotting with monoclonal antibody Ab-4.

**Phosphatase treatment.** Protein A agarose–bound immunocomplexes, obtained as indicated above, were subjected to dephosphorylation for 15 minutes at 37°C using 2 to 5 units of calf intestinal phosphatase (NEB) in 1 × NEB buffer 3. Control reactions were done by inclusion of phosphatase inhibitors (0.5 mmol/L sodium orthovanadate; 5 mmol/L p-nitrophenyl phosphatase).

**In vivo labeling of cells.** HEK 293T cells were grown in DMEM containing 10% FCS to 80% confluence and either left untreated or treated with hydroxyurea for 18 hours. MG132 was present during the last 15 hours of incubation. Cells were starved for 1 hour in phosphate-free DMEM containing 1% dialyzed FCS followed by incubation for the last 4 hours in the same medium with the addition of 1 μCi [32P]Pi. Cells were immunoprecipitated using polyclonal antibody F-15; proteins were resolved by SDS-PAGE and transferred to PVDF. Radioactive proteins were visualized using a PhosphorImager (Amersham Pharmacia) and hEXO1b was detected by Western blotting with monoclonal antibody Ab-4.

**Figure 1.** Inhibitors of DNA replication induce hEXO1b destabilization. A, Western blot analysis of hEXO1b was done using antibody F-15. Lanes 1 to 3, recombinant hEXO1b purified from *E. coli* (100, 25, and 10 ng, respectively); lanes 4 and 5, total cell lysate (50 μg) from HEK 293T cells that were left untreated or treated with hydroxyurea; lanes 6 and 7, total cell lysate (50 μg) from HEK 293T cells that were mock transfected or transfected with an expression vector encoding hEXO1b. B, HEK 293T cells were treated in the absence (lane 1) or the presence (lane 2) of hydroxyurea (2 mmol/L) for 24 hours. Endogenous hEXO1b was immunoprecipitated using polyclonal antibody F-15 and probed with monoclonal antibody Ab-4 (top). Immunoprecipitation of MSH6 from the same extracts was used as control (middle). Detection of TFIH in the whole cell extract served to ensure that equal amount of protein was used for immunoprecipitation (bottom). C, the electrophoretic migration of recombinant hEXO1b (100 ng, lane 1) or hEXO1a (125 ng, lane 2) purified from *E. coli* was compared with the migration of the protein immunoprecipitated from 293 T cells with antibody F-15, lane 3. Detection was done with monoclonal antibody Ab-4. D, HEK 293T cells were either left untreated (lane 1) or treated with 2 mmol/L hydroxyurea for 16 hours (lanes 2-8). Upon release from the hydroxyurea block (lanes 3-8), hEXO1b protein level was monitored by immunoprecipitation at the indicated time points.
DNA damage does not affect hEXO1b protein stability. To distinguish whether destabilization of hEXO1b was the result of hydroxyurea-dependent fork arrest, or rather the consequence of secondary lesions occurring at stalled forks, we examined the effect of different genotoxic agents. Treatment of HEK 293T cells with UV-C (20 J/m²), ionizing radiation (10 Gy), or cisplatin (7 μmol/L; Fig. 2A) did not reduce hEXO1b protein level, thus indicating that destabilization of hEXO1b was a specific response to replication stress. Western blot analysis of proteins involved in the DNA damage response confirmed that damage generated by genotoxic agents, such as ionizing radiation, UV-C, and cisplatin, was properly signaled (Fig. 2B). Furthermore, damage generated with the alkylating agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG) at doses that cause cell cycle arrest at G2 in a mismatch repair–dependent manner (23) did not affect hEXO1b protein level (Fig. 2C, lane 4). To further substantiate these findings, we treated cells with aphidicolin, a drug that causes stalling of the replication fork through inhibition of Pol-α and Pol-δ. As observed with hydroxyurea (Figs. 1B and 2C, lane 2), aphidicolin treatment also resulted in hEXO1b protein degradation (Fig. 2C, lane 3).

hEXO1b degradation is S phase specific and depends on inhibition of DNA replication. To rule out the possibility that the effects of hydroxyurea or aphidicolin on hEXO1b protein level would merely result from synchronization of the cells in early S phase, we examined the pattern of hEXO1b expression during transition through the cell division cycle. Analysis of hEXO1b during progression through the cell cycle, which was monitored by flow cytometric analysis (Fig. 3A), showed that hEXO1b protein was

hEXO1 could only be detected through combined immunoprecipitation with rabbit polyclonal serum F-15 followed by Western blotting using monoclonal antibody Ab-4 (Fig. 1B, top). Although monoclonal antibody Ab-4 could detect both splice variants hEXO1α and hEXO1β, expressed and purified from E. coli, the endogenous 1α isoform was undetectable upon immunoprecipitation from 293T cells (Fig. 1C, lane 3). The apparent difficulty of detecting hEXO1β in cell extracts using Western blotting was the result of low abundance of the protein in the cell (≈100 ng in 3 mg cell extract; Fig. 1C, lane 3) and low affinity of available antibodies (Fig. 1A, lanes 1-3).

hEXO1β is degraded in response to hydroxyurea. Treatment of cells with hydroxyurea, which causes replication fork arrest due to depletion of the pool of deoxynucleotides, led to clear decrease in the amount of immunoprecipitated hEXO1β protein (Fig. 1B, lane 2 versus f). Immunoprecipitation of hMSH6 from the same cell extracts was used as internal control to ensure reliability and accuracy of the procedure (Fig. 1B, middle). The correlation between hEXO1β degradation and replication fork arrest was further confirmed by time course recovery experiments where hEXO1β protein level was examined upon removal of hydroxyurea. The data showed that hEXO1β returned to regular levels within 8 hours of hydroxyurea removal (Fig. 1D), thus indicating the reversibility of the effect.
invariably present at all time points examined (Fig. 3B). We next assessed whether the destabilization of hEXO1b following hydroxyurea or aphidicolin treatment was a specific response to the inhibition of DNA synthesis or the result of other types of stress. To this end, we treated cells proceeding from M to G1, or cells in S phase with aphidicolin. The data showed that the drug caused rapid degradation of hEXO1b only if administered to S-phase cells, whereas hEXO1b protein level remained substantially constant if the treatment was done in cells progressing from M to G1 when no DNA synthesis occurs (Fig. 3C).

**hEXO1b is polyubiquitinylated in response to hydroxyurea.**

Rapid changes in cellular protein level might be the result of transcriptional, translational, or posttranslational mechanisms. To distinguish among these possibilities, we began by analyzing hEXO1b protein stability. Addition of the selective proteasome inhibitor MG132 showed an evident stabilization of hEXO1b protein in hydroxyurea-treated cells (Fig. 4A, lanes 5-8 versus lanes 1-4). These results confirmed our observation that hEXO1b was particularly unstable under conditions of stalled replication forks. To assess the involvement of ubiquitinylation in the control of hEXO1b level, we transfected HEK 293T cells with a plasmid encoding HA-tagged ubiquitin and examined hEXO1b protein upon hydroxyurea treatment. Immunoprecipitation of hEXO1b under conditions that eliminate all possible protein-protein interactions (see Materials and Methods), and therefore rule out the presence of contaminating proteins, showed that upon hydroxyurea treatment endogenous hEXO1b was polyubiquitinylated (Fig. 4B, top). This result suggested that hEXO1b degradation was mediated by ubiquitin-proteasome pathways.

**Phosphorylation of hEXO1b in response to hydroxyurea coincides with protein degradation.**

Upon close examination, we noticed that hEXO1b electrophoretic mobility was altered in hydroxyurea-treated cells compared with untreated cells (Fig. 5A, bottom, lanes 1 and 2). The retarded migration of hEXO1b was abolished upon dephosphorylation of immunoprecipitated hEXO1b with alkaline phosphatase (Fig. 5A, top and bottom, lane 3) and was left unchanged upon inclusion of phosphatase inhibitors...
(Fig. 5A, top and bottom, lane 4). To further substantiate this finding, we labeled cells with 32P. The radiolabeled isotope was added to untreated or hydroxyurea-treated cells during the last 4 hours of incubation. Immunoprecipitation experiments showed that endogenous hEXO1b was a phosphoprotein and that phosphorylation was moderately increased upon treatment with hydroxyurea (Fig. 5B, right). To address the possible link between hydroxyurea-induced hEXO1b phosphorylation and protein stability, we examined the effect of the ATM/ATR inhibitor caffeine on hEXO1b level. We observed that treatment of cells with caffeine before the addition of hydroxyurea rescued the hEXO1b protein from degradation (Fig. 5C, lane 4 versus 3). To further verify the effect of phosphorylation on hEXO1b, we used the phosphatase inhibitor okadaic acid. Addition of okadaic acid to hydroxyurea-treated cells resulted in a more pronounced decrease of hEXO1b electrophoretic mobility and drastically compromised hEXO1b stability (Fig. 5D, lane 5 versus 4). Taken together, these data suggested that a correlation existed between phosphorylation and ubiquitin-dependent degradation of hEXO1b upon hydroxyurea treatment.

**Discussion**

DNA replication and DNA repair are complex cellular processes requiring functional assembly and tight regulation of large protein complexes. DNA metabolism and the cell division cycle are highly coordinated, with checkpoint pathways acting as interface between them (24). These pathways not only ensure that DNA synthesis and repair are completed before critical transitions in the cell cycle but they are also involved in controlling the assembly of DNA repair complexes at sites of DNA damage. Defects in checkpoint control cause a wide spectrum of phenotypes, with lethality resulting from ATR or CHK1 deficiency and pathologic conditions arising from the lack of ATM or CHK2 function (25).

Here, we have examined the regulation of hEXO1b, a mismatch repair–associated exonuclease, the yeast homologue of which is responsible for generating long tracts of ssDNA at stalled replication forks (26) and unprotected telomere ends (27). Our data showed that, in synchronized cells, hEXO1b was constantly expressed throughout the cell division cycle. Given its role as DNA processing enzyme, the presence of hEXO1b in the S phase induces to speculate that it might be either present at forks but subject to tight regulation, or absent from moving forks of replication and selectively recruited upon stalling. Data obtained upon ectopic expression of hEXO1 in mouse fibroblasts (19) favor the former model, whereas evidence obtained in yeast supports the latter (28). Studies aimed at addressing the localization of endogenous hEXO1b in human cells are currently ongoing in our laboratory. Despite the evident stability of hEXO1b throughout the cell cycle, in this study we found that the level of hEXO1b was significantly reduced upon treatment of cells with hydroxyurea or aphidicolin, two agents that cause replication fork arrest by different mechanisms. Addition of aphidicolin to S-phase–synchronized cells led to rapid reduction of hEXO1b protein level. This was not the case if the drug was added to cells transiting from M to G1, thus indicating that the response to aphidicolin was not merely the result of stress but was specifically linked to DNA synthesis inhibition. Removal of the block and resumption of progression through S phase was accompanied by resynthesis of hEXO1b, suggesting that the process was fully reversible. Moreover, the fact that rapid degradation of hEXO1b was observed in response to DNA replication arrest but not upon treatment with agents that can directly induce DNA damage ruled out a role for signals from secondary lesions that may arise as consequence of fork arrest. At the molecular level, evidence obtained with the inhibitor MG132 pointed to an involvement of the proteasome in the process of hEXO1b degradation. This finding was substantiated by the appearance of polyubiquitinylated forms of hEXO1b only in hydroxyurea-treated cells. In addition to ubiquitinylation, we found that hEXO1b was a phospho-protein and that the extent of phosphorylation was increased in response to hydroxyurea. Phosphorylation of hEXO1b correlated with its rapid degradation and this was rescued by caffeine, thus implicating a role for ATM/ ATR kinases in this process. Inhibition of DNA replication in rad53-deficient yeast strains was shown to result in hemireplicated intermediates with large regions of ssDNA (29). This indicated that the integrity of stalled replication forks was maintained by tight control of an exonuclease activity in wild type, but not in rad53 strains. Colocalization studies have shown that the nucleases hMRE11 (30) and hFEN1 (31) interact with RPA and PCNA at forks of replication. These nucleases, however, are stable proteins participating in formation of nuclear foci during DNA synthesis or DNA repair (30, 32). Colocalization of ectopically expressed hEXO1 with PCNA was shown to occur in mouse fibroblasts (19). Recent evidence obtained in yeast confirmed the recruitment of exonuclease 1 to stalled forks (28). Our data show that hEXO1b is degraded in response to stalled replication and prompt to hypothesize that ATR/CHK1–dependent signaling might play a key role in the regulation of hEXO1. Such hypothesis is supported by published data showing that activation of Mec1, the S. cerevisiae ATR ortholog, occurred upon generation of long tracts of ssDNA by Exol1p (28). Future studies will aim at clarifying the possible role of ATR-dependent signals on the regulation of hEXO1b stability.

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