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Monoubiquitination of Tob/BTG family proteins competes with degradation-targeting polyubiquitination

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ABSTRACT

Tob belongs to the anti-proliferative Tob/BTG protein family. The expression level of Tob family proteins is strictly regulated both transcriptionally and through post-translational modification. Ubiquitin (Ub)/proteosome-dependent degradation of Tob family proteins is critical in controlling cell cycle progression and DNA damage responses. Various Ub ligases (E3s) are responsible for degradation of Tob protein. Here, we show that Tob family proteins undergo monoubiquitination even in the absence of E3s *in vitro*. Determination of the ubiquitination site(s) in Tob by mass spectrometric analysis revealed that two lysine residues (Lys48 and Lys63) located in Tob/BTG homology domain are ubiquitinated. A mutant Tob, in which both Lys48 and Lys63 are substituted with alanine, is more strongly polyubiquitinated than wild-type Tob *in vivo*. These data suggest that monoubiquitination of Tob family proteins confers resistance against polyubiquitination, which targets proteins for degradation. The strategy for regulating the stability of Tob family proteins suggests a novel role for monoubiquitination.

1. Introduction

Tob, a member of the anti-proliferative Tob/BTG family of proteins, is involved in the regulation of cell cycle progression at the G1 phase [1–3]. Tob's expression levels and phosphorylation status change during the cell cycle [2], and decreased expression and/or phosphorylation relative to normal tissues have been detected in several human cancers, including lung and thyroid cancer [3,4]. These observations suggest that the level of Tob expression and its phosphorylation status are strictly regulated to maintain normal cell growth. Whereas the expression of Tob family genes is regulated at the transcriptional level during cell differentiation or DNA damage response [1], it has been reported that post-translationally the Ub/proteosome pathway is also involved in controlling the amounts of these proteins [5]. Indeed, several E3 Ub ligases including SCF^{Skp2} regulate the level of Tob protein [6,7]. Furthermore, proteosome-mediated degradation of Tob is important for regulating cell cycle progression as well as DNA damage-induced apoptosis [6,8].

Proteins targeted for degradation by the Ub/proteosome system are marked by covalent linkage to Ub. Initially, Ub is activated by a Ub-activating enzyme (E1) in an ATP-dependent manner. The activated Ub is subsequently transferred to a Ub-conjugating enzyme (E2), and ultimately binds to a lysine residue on the substrate in a process mediated by an E3 ligase [9]. Different types of Ub modifications have distinct cellular functions [10–12]. A polyUb chain linked to Lys48 is associated with proteosome-dependent degradation, whereas a polyUb chain linked to Lys63 affects endocytosis or DNA repair processes. Moreover, covalent modification of proteins by a single Ub (monoubiquitination) plays important roles in regulation of signal transduction, endocytosis, and DNA repair processes [13,14]. Recent reports have shown a number of Ub-binding domain (UBD)-containing proteins to be monoubiquitinated *in vitro* independently of the activity of E3 ligases [15]. Monoubiquitination of ubiquitin-binding proteins inhibits their capacity to bind to and control the functions of ubiquitinated targets *in vivo* [16].

Abbreviations: Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; UBD, ubiquitin-binding domain; PCR, polymerase chain reaction; GST, glutathione S-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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In this study, we found that Tob/BTG family proteins are monoubiquitinated in the absence of E3s *in vitro*. We further showed that the monoubiquitination event correlates with lower levels of polyubiquitination. Therefore, monoubiquitination of Tob/BTG family proteins appears to regulate their stability by affecting polyubiquitination and concomitant proteosome-dependent degradation.

2. Materials and methods

2.1. DNA constructs

The cDNAs encoding Tob family genes were inserted into pET-26b expression vector (Novagen) for protein expression in *Escherichia coli*. Since full-length Tob protein could not be prepared due to its instability and insolubility, Tob constructs lacking several amino acids in the carboxy-terminal region were prepared. The Tob deletion constructs (amino acids 1–332, 1–232 and 1–112) were constructed by polymerase chain reaction (PCR). The cDNAs of E2 proteins were cloned by PCR and inserted into pGEX6P-1 vector (GE Healthcare). To express Tob in mammalian cells, we used the SRα promoter-driven mammalian expression vector pME18S [2].

2.2. Preparation of recombinant proteins

6xHis-tagged proteins of the Tob/BTG family were expressed in *E. coli* BL21, and the cells were lysed by sonication in buffer A (50 mM Tris–HCl [pH 8.0], 200 mM NaCl, 1 mM PMSF, 5 mM imidazole). The proteins were purified using Ni–NTA Sepharose beads (Qiagen), and eluted from the beads with buffer A containing 125 mM imidazole. Glutathione S-transferase (GST)-fused E2 proteins expressed in BL21 *E. coli* were purified with glutathione-Sepharose beads (GE Healthcare). The GST portion of each protein was cleaved with precision protease (GE Healthcare). All purified proteins were dialyzed in buffer B (20 mM Tris–HCl [pH 7.5], 50 mM NaCl, 5% glycerol).

2.3. Antibodies

Anti-Tob monoclonal antibody (4B1) was obtained from IBL. Anti-ubiquitin (FK2) and anti-His (OGHis) antibodies were purchased from MBL.

2.4. Cell culture, transfection, and immunoblotting

Cos7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. The cells were transfected with the expression plasmids using FuGENE 6 transfection reagent (Roche). Immunoblot analysis was performed as described previously [2].

2.5. Detection of polyubiquitination in vivo

Cos7 cells were co-transfected with Tob expression vectors and 6xHis-Ub expression vector. The cells were treated the next day with 50 μM MG132 (Peptide Institute) for 3 h. Cell lysates were prepared using denaturing buffer (6 M guanidine–HCl, 10 mM phosphate buffer [pH 8.0], 10 mM imidazole). His-Ub-conjugated proteins were purified with Ni–NTA agarose and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting.

2.6. In vitro ubiquitination assay

Bovine ubiquitin (15 μg, Sigma), recombinant E1 (200 ng, Boston Biochem), recombinant E2 protein (1 μg), and recombinant Tob family proteins (100 ng) were mixed in 30 μl of buffer C (40 mM Tris–HCl [pH 7.4], 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 μM ATP). The mixtures were incubated for 1.5 h at 30 °C, and the reaction products were analyzed by SDS–PAGE followed by immunoblotting.

2.7. Mass spectrometric analysis

The *in vitro* ubiquitination of Tob was performed as described above. In total, 5 μg of recombinant Tob was subjected to the reaction. After the reaction, Tob was purified with Ni–NTA Sepharose in buffer (20 mM Tris–HCl [pH 7.5], 500 mM NaCl, 1% Triton X-100, 5 mM 2-mercaptoethanol). The purified Tob was then separated by SDS–PAGE and visualized using Silver Staining Kit MS (Wako). For mass spectrometric analysis, bands corresponding to ubiquitinated Tob were excised from the gel and digested in the gel with trypsin (1 pmol/μl) for 12 h at 37 °C. The eluted peptides were loaded onto the automated nanoflow liquid chromatograph (Dina) and tandem mass spectrometer (QSTAR Elite). The peptide masses obtained by LC–MS/MS analysis were searched against the non-redundant protein sequence database of the National Center for Biotechnology Information using the Mascot search engine (Matrix Science, London, UK).

3. Results

3.1. In vitro monoubiquitination of Tob

Tob/BTG family proteins are polyubiquitinated and subsequently degraded by the 26S proteosome [5,8]. Several E3 Ub ligases are responsible for Tob ubiquitination [6,7]. To detect the activity of Tob ubiquitination in cytoplasmic extracts, we set up an *in vitro* ubiquitination assay using recombinant E1, E2 and Tob-His (amino acids 1–332) as well as Ub. Unexpectedly, a single slow migrating form of Tob was detected by immunoblotting even in reactions lacking cytoplasmic extracts (Fig. 1, left and middle panels). The slow migrating form was recognized by the anti-Ub antibody and was produced only when all the protein components were present (Fig. 1, right panel). We did not detect any obvious signals other than the slow migrating form. These data suggest that Tob is monoubiquitinated *in vitro* in a manner independent of E3s.

3.2. Tob/BTG family proteins are monoubiquitinated in vitro

We next carried out an *in vitro* ubiquitination assay using the two shorter forms of His-tagged Tob, Tob-His (1–232) and Tob-His (1–112), and found both forms were also monoubiquitinated (Fig. 2A). As the amino acid sequence (amino acids 1–112) is well conserved among the Tob/BTG family proteins, we tested whether the propensity to undergo monoubiquitination is conserved among protein family members. *In vitro* ubiquitination assay followed by immunoblot analysis revealed that both Tob2 and BTG2 also became monoubiquitinated (Fig. 2B). Interestingly, UBCH4 and UBCH5a but not UBCH3 were able to mediate the monoubiquitination of Tob/BTG family proteins.

3.3. Determination of monoubiquitination site(s)

The data shown in Fig. 2A indicate that monoubiquitination of Tob occurs within its N terminal half. To determine the monoubiquitination site(s), we performed mass spectrometry. We prepared large amounts of *in vitro* ubiquitinated Tob proteins and subjected them to SDS–PAGE (Fig. 3A). After silver staining of the gels, the corresponding band was cut out from the gel and analyzed by peptide mass fingerprinting, and we identified Lys48 and Lys63 of Tob