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Detection of O-propargyl-puromycin with SUMO and ubiquitin by click chemistry at PML-nuclear bodies during abortive proteasome activities

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ABSTRACT

The amino-nucleoside antibiotic, puromycin, acts by covalently linking to elongating polypeptide chains on ribosomes to generate prematurely terminated immature polypeptides. The trafficking of puromycin-conjugated (puromycylated) immature polypeptides within cell has, however, remained elusive. In this study, using O-propargyl-puromycin (OP-Puro), the distribution of puromycylated polypeptides was assessed in HeLa cells by click chemistry. Under standard culture conditions, OP-Puro signals were detected in the cytoplasm and nucleus with the highest concentrations in the nucleolus. Intriguingly, when proteasome activities were aborted using MG132, OP-Puro signals began to accumulate at promyelocytic leukemia nuclear bodies (PML-NBs) in addition to the nucleolus. We also found promiscuous association of OP-Puro signals with SUMO-2/3 and ubiquitin at PML-NBs, but not at the nucleolus, during abortive proteasome activities. This study reveals a previously unknown distribution of OP-Puro that argues for a nuclear function in regulating immature protein homeostasis.

1. Introduction

Puromycin, an amino-nucleoside antibiotic produced by *Streptomyces alboniger*, is a potent protein synthesis inhibitor in both prokaryotic and eukaryotic cells [1]. Puromycin enters the ribosome A site and terminates translation by ribosome-catalyzed covalent incorporation into the nascent polypeptide chain, referred to as 'puromycylation' [2–8]. Because this generates prematurely terminated and misfolded polypeptides, puromycin-conjugated (puromycylated) polypeptides are likely to cause cytotoxic effects and thus may contribute to induction of the stress response and growth defects; however, regulation of their trafficking and fate in vital cellular systems remain largely uncharacterized.

Promyelocytic leukemia-nuclear bodies (PML-NBs), also known as nuclear dot 10 (ND10) or PML oncogenic domains (PODs), are nuclear domains characterized by the presence of the tripartite motif family protein PML/TRIM19 [9–13]. PML-NBs are involved in a wide variety of cellular activities, including the stress response, aging and viral infection [9–13]. A wide variety of proteins are assembled into PML-NBs via post-translational modification of PML and/or its interactors mediated by the SUMO-2/3 and ubiquitin conjugation pathways. This may contribute to a protective response involving the intra-nuclear sequestration, storage or degradation of non-functional and aberrant proteins [9–13].

Here we use a puromycin derivative, O-propargyl-puromycin (OP-Puro) to monitor puromycylated polypeptides in cultured HeLa cells. OP-Puro is incorporated into elongating polypeptides on ribosomes in a manner similar to puromycin and can be fluorescently labeled by an azide-alkyne reaction, called 'click chemistry' [4–8]. This enables the subcellular distribution of OP-puromycylated polypeptides to be visualized in cells. Notably, PO-Puro signals are detected in the cytoplasm and nucleus with substantial concentration at the nucleolus under standard culture conditions. Intriguingly, OP-Puro signals are detected at multiple small nuclear foci when the proteasome inhibitor MG132 is co-administrated. These small nuclear foci may represent PML-NBs, because PML,

Abbreviations: OP-Puro, O-propargyl-puromycin; SUMO, small ubiquitin-related modifier; PML-NBs, promyelocytic leukemia-nuclear bodies; DAPI, 4′,6-diamidino-2-phenylindole.

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SUMO-2/3 and ubiquitin co-accumulate at the foci. Our findings suggest a role for the nucleus together with the SUMO and ubiquitin pathways in protein homeostasis and the intracellular sequestration of immature polypeptides.

2. Materials and methods

2.1. Cell culture and drug treatments

HeLa cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham with 5% fetal calf serum and antibiotics at 37 °C in 5% CO_2 incubator. Protein synthesis inhibitors, puromycin, cycloheximide, emetin and anisomycin, were obtained from Wako Pure Chemical Industries. O-propargyl-puromycin (OP-Puro) was obtained from Jena Bioscience. Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (MG132) was purchased from Peptide Institute Inc. Each drug was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium as indicated in the text.

2.2. Antibodies

Anti-SUMO-2/3 and -SUMO-1 rabbit monoclonal antibodies were obtained from Cell Signaling Technologies. Anti-poly-ubiquitin mouse monoclonal (FK2), anti-PML and anti-nucleolin antibodies were obtained from Medical and Biological Laboratories (MBL), Santa Cruz Biotechnology and MerckMillipore, respectively. Secondary antibodies used were purchased from Jackson ImmunoResearch, Life technologies and MBL.

2.3. OP-Puro labeling of cultured HeLa cells and detection by fluorescence microscopy

OP-Puro labeling was carried out as previously described [5]. In brief, HeLa cells were grown on glass coverslips in culture medium. To visualize OP-Puro incorporation, we typically exposed cells to culture medium containing 10 μM OP-Puro and 10 μM MG132 for 4 h at 37 °C. The cells were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were washed with PBS, followed by permeabilization with PBS containing 0.1% Triton X-100, and then washed with PBS. OP-Puro was detected by performing an azide-alkyne cycloaddition for 30 min at room temperature using the Click-iT Cell Reaction Buffer Kit containing 15 μM Alexa 488/555-N_3 azide (Life Technologies). After staining, the coverslips were washed several times with PBS and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). If indirect-immunofluorescence was performed using specific antibodies to co-localize OP-Puro signals, the cells were incubated with the appropriate antibody and the indirect-immunofluorescence reaction continued as described below. Fluorescence images were obtained using a DP72 microscope (Olympus).

2.4. Indirect-immunofluorescence analysis

Indirect-immunofluorescence was performed as described previously [14,15]. Briefly, HeLa cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were blocked in PBS containing 0.2% bovine

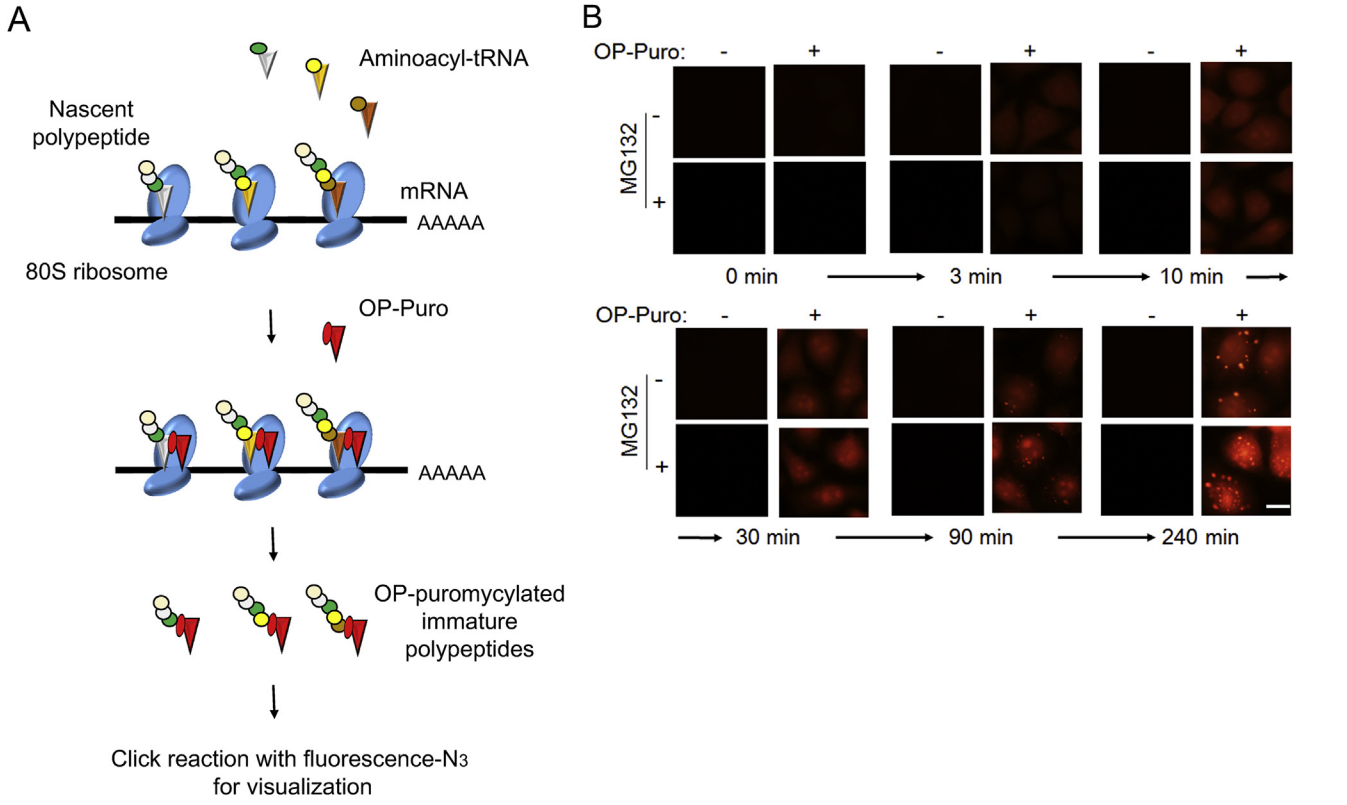

Fig. 1. OP-Puro signals accumulated in the nucleus under standard conditions, and were enhanced during abortive proteasome activities. (A) Schematic representation of the effects of OP-Puro/puromycin. OP-Puro/puromycin, leads to premature release of nascent polypeptides by puromycylation and thus inhibiting polypeptide chain elongation on the ribosome and releasing puromycylated immature polypeptides. In the case of OP-puromycylated polypeptides, their subcellular localization can be visualized by click reaction with fluorescence-conjugated N_3. B) Exponentially growing HeLa cells under standard culture conditions were exposed to 10 μM Puro in the absence (upper panels) or presence (bottom panels) of 10 μM MG132 for the indicated periods at 37 °C, and were then subjected to the click reaction using Alexa 488-N_3 to visualize OP-Puro localization. Bar indicates 20 μm.