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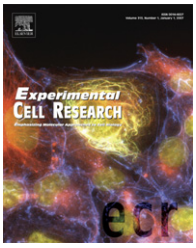

Research Article

14-3-3-dependent inhibition of the deubiquitinating activity of UBPY and its cancellation in the M phase

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ABSTRACT

The deubiquitinating enzyme UBPY, also known as USP8, regulates cargo sorting and membrane traffic at early endosomes. Here we demonstrate the regulatory mechanism of the UBPY catalytic activity. We identified 14-3-3 ε, γ, and ζ as UBPY-binding proteins using co-immunoprecipitation followed by mass spectrometric analysis. The 14-3-3 binding of UBPY was inhibited by mutating the consensus 14-3-3-binding motif RSYS^{680}SP, by phosphatase treatment, and by competition with the Ser^{680}-phosphorylated RSYS^{680}SP peptide. Metabolic labeling with [^{32}P]orthophosphate and immunoblotting using antibody against the phosphorylated 14-3-3-binding motif showed that Ser^{680} is a major phosphorylation site in UBPY. These results indicated that 14-3-3s bind to the region surrounding Ser^{680} in a phosphorylation-dependent manner. The mutation at Ser^{680} led to enhanced ubiquitin isopeptidase activity of UBPY toward poly-ubiquitin chains and a cellular substrate, epidermal growth factor receptor, in vitro and in vivo. Moreover, addition of 14-3-3ε inhibited the UBPY activity in vitro. Finally, UBPY was dephosphorylated at Ser^{680} and dissociated from 14-3-3s in the M phase, resulting in enhanced activity of UBPY during cell division. We conclude that UBPY is catalytically inhibited in a phosphorylation-dependent manner by 14-3-3s during the interphase, and this regulation is cancelled in the M phase.

Introduction

Ubiquitination is a post-translational protein modification that is mediated by the ubiquitin (Ub)-conjugating system composed of E1, E2, and E3 enzymes. It serves as diverse signals that regulate the fate and function of a number of intracellular proteins. Ubiquitination is a reversible modification, as conjugated Ub molecules can be removed from target proteins by deubiquitinating enzymes (DUBs). The human genome encodes nearly 100 DUBs, and their cellular functions have just begun to be elucidated in the last several years [1,2].

Cell surface receptor proteins that have undergone endocytosis are first delivered to the early endosome. Among them, nutrient receptors such as the transferrin receptor are thereafter recycled back to the plasma membrane. By contrast, ligand-activated signaling receptors such as receptor tyrosine kinases are further transported to lysosomes for degradation. Early endosomes sort these two types of cargoes depending on whether they are ubiquitinated. Namely, ubiquitination in the cytoplasmic regions of cargo proteins serves as a sorting signal for selective transport to lysosomes [3,4]. On the endosomal membrane, ubiquitinated cargoes are initially recognized by a complex of two Ub-binding proteins, Hrs and STAM. Subsequently, the cargoes are transferred to three protein complexes, ESCRT (endosomal sorting complex required for transport)-I, II, and III, and incorporated into lumenal vesicles of endosomes

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that bud inwardly from their limiting membrane. Following fusion of such vesicle-containing endosomes, called multivesicular bodies, with lysosomes delivers the sorted cargoes to the lumen of lysosomes. In this process, not only cargoes but also some sorting machinery proteins including Hrs and STAM undergo ubiquitination [5,6]. Therefore, the lysosomal trafficking of ubiquitinated cargoes is likely to be regulated by ubiquitination of the sorting machinery proteins [7].

UBPY, also known as USP8, is a DUB that belongs to the Ub-specific protease family of cysteine proteases [8]. The Hrs-STAM endosomal sorting complex interacts with UBPY via the Src homology 3 domain of STAM [9]. Two functions have been demonstrated for UBPY on endosomes. First, it deubiquitinates ligand-activated and endocytosed epidermal growth factor receptor (EGFR), a cargo that is sorted for lysosomal trafficking in a ubiquitination-dependent manner, on early endosomes [10]. By removing the lysosomal sorting signal, UBPY negatively regulates the downregulation of EGFR [10]. However, there also are reports that deubiquitination by UBPY is required for the lysosomal trafficking of activated EGFR [11,12]. Second, UBPY deubiquitinates various unidentified proteins on the early endosome membrane, and the UBPY-mediated regulation of the level of protein ubiquitination is crucial for maintaining the morphology and function of early endosomes [12,13].

The 14-3-3 family of proteins are highly-conserved ubiquitous proteins in eukaryotic cells [14,15]. In mammals, there are seven members: β, ε, γ, η, σ, τ, and ζ. 14-3-3 proteins often bind to short phospho-Ser-containing amino acid motifs, most commonly RSXpSXP where pS represents the phospho-Ser residue, in a variety of intracellular proteins [16,17]. The binding of 14-3-3s regulates the functions of target proteins in several ways [14,15]. It elevates or inhibits the catalytic activity of target enzymes by changing their conformation. In other cases, it stabilizes the phosphorylated state of target proteins by blocking the access of protein phosphatases to the phospho-Ser residue in the 14-3-3-binding motif. It also regulates the subcellular localization and trafficking of target proteins by masking the localization and retention signals within the proteins. 14-3-3 proteins normally exist as homo- or hetero-dimers [18]. Therefore, by binding to two different proteins simultaneously, the 14-3-3 dimer also serves as an adaptor that couples two target proteins.

The mechanisms underlying the regulation of the DUB activity are mostly unknown. Here, we show that the catalytic activity of UBPY is inhibited by Ser^{680}-phosphorylation-dependent binding of 14-3-3s to a consensus 14-3-3-binding motif surrounding Ser^{680}. We further show that this inhibition is cancelled during cell division.

Materials and methods

cDNA expression constructs and transfection

Expression vectors for FLAG-tagged mouse UBPY and $UBPY^{C748A}$ were constructed as described [9]. Expression vectors for FLAG-tagged mouse $UBPY^{R677A}$, $UBPY^{S678A}$, $UBPY^{S680A}$, $UBPY^{P682A}$, and $UBPY^{S680A/C748A}$ were constructed using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA), and their DNA sequences were verified. Construction of the human EGFR expression vector was described previously [19]. Expression vectors for FLAG-tagged mono-Ub and c-Cbl were provided by Dr. T. Suzuki (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and Dr. K. Yokote (Chiba University, Chiba, Japan), respectively. Expression vectors were transfected into cells using the FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN).

Immunoprecipitation and immunoblotting

Cell lysates were prepared by solubilizing cells with lysis buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) for 30 min on ice and collecting the supernatant after centrifugation at 12,000×*g* for 15 min at 4 °C. The lysates were used for immunoblotting, or immunoprecipitated with anti-FLAG M2 (1 μg, Sigma-Aldrich, St. Louis, MO), anti-EGFR (1 μg, MBL, Nagoya, Japan), and anti-UBPY (7 μl) [9] antibodies. The immunoblot analysis was performed using standard procedures. The primary antibodies used were anti-FLAG M2 (4 μg/ml), anti-14-3-3β (1 μg/ml, H-8, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-14-3-3-binding motif (1:4,000, Cell Signaling Technology, Danvers, MA), anti-Ub (1 μg/ml, Covance, Princeton, NJ), anti-UBPY (1:500) [9], anti-EGFR (0.5 μg/ml, MBL), anti-cyclin B (1 μg/ml, Santa Cruz Biotechnology), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (1:1000, Cell Signaling Technology), and anti-α-tubulin (1 μg/ml, Sigma-Aldrich) antibodies. Secondary antibodies were peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (GE Healthcare, Piscataway, NJ). Blots were detected using the ECL reagent (GE Healthcare).

To examine cells stimulated with EGF, cells were cultured in the presence of 0.5% fetal bovine serum for 24 h and incubated with EGF (100 ng/ml, PeproTech, Rocky Hill, NJ) for a given period at 37 °C before lysates were prepared.

Mass spectrometry

The lysate of HeLa cells transfected with FLAG-UBPY was immunoprecipitated with agarose beads conjugated with anti-FLAG antibody (anti-FLAG M2 affinity gel, Sigma-Aldrich). FLAG-UBPY was eluted from the beads by incubation with 100 μl of phosphate-buffered saline (PBS) containing the FLAG peptide (150 μg/ml, Sigma-Aldrich). Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), visualized by silver staining (Silver Stain MS kit, Wako, Osaka, Japan), and excised from the gel. In-gel digestion with trypsin (Promega, Madison, WI) followed by analyses using UltraFlex matrix-assisted laser desorption ionization/time of flight mass spectrometry (Bruker Daltonics, Billerica, MA) was performed according to the manufacturer's instructions. The peptide mass fingerprinting data were analyzed using the MASCOT search program (Matrix Science, Boston, MA).

Glutathione S-transferase (GST) pull-down

The 14-3-3ε cDNA was amplified by PCR from the Marathon-Ready mouse brain cDNA library (BD Biosciences Clontech,