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1 Short communication

2 Sirtuin 3 interacts with Lon protease and regulates its acetylation status

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ABSTRACT

Q2 Lon is a mitochondrial protease that degrades oxidized damaged proteins, assists protein folding and participates 20 in maintaining mitochondrial DNA levels. Changes in Lon mRNA levels, protein levels and activity are not always 21 directly correlated, suggesting that Lon could be regulated at post translational level. We found that Lon and 22 SIRT3, the most important mitochondrial sirtuin, colocalize and coimmunoprecipitate in breast cancer cells, 23 and silencing or inhibition of Lon did not alter SIRT3 levels. Silencing of SIRT3 increased the levels of Lon protein 24 and of its acetylation, suggesting that Lon is a target of SIRT3, likely at K917. 25

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Q3 1. Introduction

32 Lon protease (hereafter indicated as Lon) is a highly conserved mitochondrial protein which occupies a key position in mitochondrial functions. Lon is encoded by the nuclear gene PRSS15. The mature enzyme is a 959-aa protein composed of three domains (Garcia-Nafria et al., 2010; Wang et al., 1993): the N-terminal domain, which interacts with protein substrates, the AAA+ module, involved in ATP binding and hydrolysis, and the P domain that includes the active site necessary for proteolytic activity (Matsushima et al., 2010). Lon enzymatic activity is stimulated by ATP, and inhibited by 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and some derivatives (Bernstein et al., 2012). In mitochondria, Lon degrades oxidized and damaged proteins, acts as a chaperon, by assisting the assembly and folding of mitochondrial proteins, and participates in the maintenance and replication of mtDNA. So far, only few substrates of Lon have been identified in humans, including aconitase, TFAM, Cox4-1, StAR, and ALAS-1 (Bota and Davies, 2002; Fukuda et al., 2007; Granot et al., 2007; Ondrovicova et al., 2005; Tian et al., 2011). Lon levels and proteolytic activities are regulated by several cellular stressors, such as reactive oxygen species (ROS) and low oxygen tension (Fukuda et al., 2007; Ngo and Davies, 2009; Pinti et al., 2011; Pinti et al., 2010). Most of these stressors act by increasing the transcription of PRSS15. 52

53 Almost one-third of all proteins within mitochondria are acetylated, of which the majority are involved in important metabolic pathways, thus highlighting the critical role of this post-translational modifications for cellular homeostasis (Zhao et al., 2010). However, the effect of acetylation on most of these proteins is unknown. The acetylation state of mitochondrial proteins is controlled by the NAD+-dependent mitochondrial deacetylase sirtuin 3 (SIRT3) (Onyango et al., 2002). SIRT3-mediated deacetylation reaction is characterized by lysine deacetylation and NAD hydrolysis, thus yielding 2′-O-acetyl-ADP ribose, the deacetylated substrate, and nicotinamide. Several SIRT3 targets have been identified, including glutamate dehydrogenase, isocitrate dehydrogenase 2, and manganese superoxide dismutase; deacetylation of these proteins leads to an increase of their enzymatic activity (Hirschey et al., 2010; Rauh et al., 2013; Tao et al., 2010). 66

Abbreviations: Lon, Lon protease; CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; PMSF, phenylmethanesulfonyl fluoride; SIRT3, sirtuin 3; AADPR, 2′-O-acetyl-ADP ribose; DMEM, Dulbecco's modified Eagle's medium; NAD, nicotinammide adenina dinucleotide; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PBS, phosphate buffer saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DABCO, 1,4-diazabicyclo [2.2.2]octane; EDTA, Ethylenediaminetetraacetic acid; NaF, sodium fluoride.

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Here we show that SIRT3 interacts with Lon, and that SIRT3 silencing causes an increase of Lon levels and acetylation, suggesting that Lon is a substrate of SIRT3 deacetylase activity.

2. Materials and methods

2.1. Cell culture

MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Corporation) supplemented with 10% foetal bovine serum and gentamycin, at 37 °C in 5% CO_2. For hypoxic treatments, cells were incubated in the hypoxia chamber (STEMCELL technologies, Vancouver, Canada) in the presence of 1% O_2, 5% CO_2 and 94% N_2 at 37 °C. When indicated, oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO, a specific inhibitor of Lon) was added to cell culture for 24 h at concentrations 0.1 and 1 μM.

2.2. Isolation of mitochondria

Mitochondria were isolated according to a revised version of the protocol described in (Ahmed et al., 2010; Nyblom et al., 2006). Cells were washed with PBS, trypsinized and resuspended in ice-cold homogenization buffer (0.25 M sucrose, 0.25 mM EGTA, 5 mM HEPES, 1 mM DTT and protease inhibitor cocktail, pH 7.4). Cells were then disrupted by 10 strokes of a Teflon pestle in a glass homogenizer. The homogenization and subsequent steps were performed on ice. The homogenates were centrifuged at 1000 ×g for 10 min. The pellet was rehomogenized and centrifuged at 1000 ×g. The combined supernatants were centrifuged at 17,000 ×g for 15 min. The mitochondria pellet was washed again with 1 mL of PBS, centrifuged, frozen in liquid nitrogen and stored at −80 °C. All reagents were from Sigma Aldrich (St. Louis, MO, USA).

2.3. Immunoprecipitation

Briefly, whole cells or mitochondrial fractions were extracted by the addition of RIPA buffer (20 mM Tris-Cl, pH 7.0; 1% Nonidet P-40; 150 mM NaCl; 10% glycerol; 10 mM EDTA; 20 mM NaF; 5 mM sodium pyrophosphate; and 1 mM Na_3VO_4) and freshly added Sigma-Aldrich Protease Inhibitor Cocktail at 4 °C for 30 min. Lysates were sonicated, cleared by centrifugation and used for immunoprecipitation experiments, as described below. Equal amounts of precleared lysates were incubated at 4 °C overnight with primary antibody (Ab) followed by a 1 hour incubation with 30 μl of 50% (v/v) protein A/G agarose (GE Healthcare, Little Chalfont, United Kingdom) with agitation, as described previously (Bertacchini et al., 2013). Pellets were washed three times in RIPA buffer, once with wash buffer (50 mM Tris, pH 8) and boiled in Laemmli sample buffer. Immunoprecipitates and proteins from whole cells or mitochondrial fractions were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, blotted with indicated Ab, and detected using a chemiluminescence method.

2.4. Confocal microscopy

Cells were grown on glass coverslips. After treatment, cells were washed with PBS, and fixed with 4% paraformaldehyde in PBS for 15 min. Fixed monolayer cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 3% BSA in PBS for 30 min at room temperature. Then, samples were incubated with primary Abs in PBS containing 3% BSA, for 1 h at room temperature. After washing in PBS containing 3% BSA, samples were incubated for 1 h at room temperature with secondary Abs. After washing in PBS samples were stained with 1 μg/ml DAPI in PBS for 1 min, and then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0). Negative controls were samples not incubated with the primary Ab. The multi-labelling immunofluorescence experiments were carried out avoiding cross-reactions between primary and secondary Abs. Fluorescent samples were observed by a Nikon A1 confocal laser scanning microscope (Nikon, Tokio, Japan).

2.5. In silico analysis

K917 is located on the surface of the catalytic domain of the human Lon protease (PDB_ID: 2X36, (Garcia-Nafria et al., 2010)). The acetylation of K917 was introduced in the 3D protein model with the Molefacture tool (http://www.ks.uiuc.edu/Research/vmd/), as previously described for other proteins (Pierri et al., 2014). In order to study the possible deacetylation of K917, a protein–protein docking analysis was performed by using the catalytic domain of the human Lon, hosting the acetylated K917, and the 3D structure of SIRT3 (PDB_ID: 4FVT). The SIRT3 crystallized structure (PDB_ID: 4FVT) contains a carba-NAD molecule and the Ac-ACS peptide with an acetylated lysine (Szczepankiewicz et al., 2012). A NAD+ cofactor molecule was obtained from carba-NAD+ by using the Molefacture tool (http://www.ks.uiuc.edu/Research/vmd/) (Humphrey et al., 1996). Protein inspection was performed by using PyMOL and the WhatIF tools in order to verify that the modified cofactor has not introduced any clush with close residues of the 3D protein model (Vriend, 1990). The GRAMM-X webserver was used for the protein docking analysis (Pierri et al., 2010). The region including residues from 280 to 300 (most of them involved in the binding of the Ac-ACS peptide) was screened for building/evaluating the best interactions with the acetylated K917 of Lon. In order to validate protein–protein docking analysis the complex obtained from the GRAMM-X webserver was inspected by using VMD and PyMOL in order to compare the binding region of the acetylated-K917 of the Lon protease with the binding region of the acetylated peptide crystallized in complex with SIRT3 (PDB_ID: 4FVT). Furthermore, a 3D complex containing the deacetylated Lon was obtained by superimposing the original docked complex with the deacetylated K917 (contained in the Lon 3D model) and a further crystallized structure of SIRT3 that contains (among other ligands) the 2′-O-acetyl-ADP-ribose (PDB_ID: 4BVH) that should be obtained following the K917 deacetylation and NAD+ degradation. Nicotinamide ligand was introduced in the second protein complex model by duplicating the nicotinamide ring from the NAD+ molecule docked within the first 3D protein complex (i.e. the ones obtained by Carba-NAD) by using PyMOL tools (Bota and Davies, 2002). One hundred steepest descent minimization steps were performed on both the protein–protein complexes obtained as described above by using VegaZZ (Pierri et al., 2010). Experimentally detected post-translational modifications of Lon proteases were recovered from the PhosphoSitePlus resource by indicating Lon within Protein name field (http://www.phosphosite.org/proteinAction.do?id=11810&showAllSites=true).

2.6. Small interfering RNA (siRNA) transfection

Cells were transfected with siRNA using RNAiMax reagent (Life Technologies Corporation) according to the manufacturer's protocol. In brief, cells were reverse transfected with 10 nM siRNA against the Lon mRNA (namely s17901, s17902, s17903 by Life Technologies Corporation) and SIRT3 mRNA (namely s23766 by Life Technologies Corporation), and siRNA Negative Control (Life Technologies Corporation) was used as a control. Cells were incubated for 48 h or 72 h to quantify mRNA and protein levels, respectively.

3. Results and discussion

We previously noticed that, despite the fact that Lon is regulated at the promoter level (11), clear discrepancies exist between mRNA and protein levels in different stress conditions. Thus, we wondered whether, within mitochondria, Lon is regulated by other mechanisms, and in particular by acetylation. Indeed, almost one-third of mitochondrial proteins are acetylated. While acetylation within mitochondria is

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