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SUMO-2/3 conjugates accumulating under heat shock or MG132 treatment result largely from new protein synthesis ☆

Markéta Častorálová, Dagmar Březinová, Martin Švéda, Jan Lipov, Tomáš Ruml, Zdeněk Knejzlík *

Department of Biochemistry and Microbiology and Center of Applied Genomics, Institute of Chemical Technology, Prague, Technická 5, 16628 Prague, Czech Republic

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ABSTRACT

Small ubiquitin-related modifiers 1, 2 and 3 (SUMO-1, -2, -3), members of the ubiquitin-like protein family, can be conjugated to various cellular proteins. Conjugates of SUMO-2 and SUMO-3 (SUMO-2/3) accumulate in cells exposed to various stress stimuli or to MG132 treatment. Although the proteins modified by SUMO-2/3 during heat shock or under MG132 treatment have been identified, the significance of this modification remains unclear. Our data show that the inhibition of translation by puromycin or cycloheximide blocks both the heat shock and MG132 induced accumulation of SUMO-2/3 conjugates in HEK 293T and U2OS cells. However, the heat shock induced accumulation of SUMO-2/3 conjugates was restored by proteasome inhibition, which suggests that the inhibition of translation did not abolish SUMOylation itself. Furthermore, we show that some of the proteins truncated due to the treatment by low concentration of puromycin are SUMOylated in HEK 293T cells. We suggest that the SUMO-2/3 conjugates accumulating under the heat shock or MG132 treatment result largely from new protein synthesis and that portion of them is incorrectly folded.

1. Introduction

Small ubiquitin-related modifier (SUMO) is covalently attached to protein targets by similar mechanism as ubiquitin and other members of ubiquitin-like proteins group I. Yeast SUMO is encoded by a single gene (*smt3*), while vertebrates have three independently encoded SUMO isoforms. Human SUMO-1 exhibits approximately 51% sequence identity to human SUMO-2 and SUMO-3 isoforms. Processed SUMO-2 exhibits approximately 97% sequence identity to processed SUMO-3 (both together designated further as SUMO-2/3). However, common enzymatic components are involved in conjugation of all three SUMO isoforms [1,2]. SUMO molecules are activated by a nuclear [3,4] heterodimeric complex Aos1/Uba2 in an ATP-dependent manner and then transferred to the Cys^{93} located in an active site of Ubc9 (reviewed in [5]), which is the only known SUMO conjugating enzyme. Ubc9 alone recognizes protein targets via short amino acid motif with consensus sequence ΨKxE/D (Ψ — amino acid with hydrophobic bulky side chain, x — any amino acid) and catalyzes transfer of SUMO molecules to the internal lysine residue of target proteins. Some proteins are modified in non-consensus sites [6–9], but the mechanism of their recognition is still not well understood. Although Ubc9 alone is able to SUMOylate most cellular protein targets, E3 SUMO ligases, such as RanBP2 [10,11], Pc2 [12,13], Topors [14] and PIAS [15,16], are necessary for higher SUMOylation efficiency and selectivity. SUMO attached to a protein target can induce its conformational changes, sterically inhibit its DNA binding activity, modulate protein–protein interaction or increase stability of the target by blocking lysine residue from ubiquitylation [17]. The SUMO attachment is a reversible modification, the isopeptide bond between SUMO and targets can be cleaved by SUMO proteases (Ulp1 and Ulp2 in yeast, SENP1, 2, 3, 5, 6 and 7 in vertebrates, reviewed in [18]).

Proteomic studies showed that some targets are selectively modified by SUMO-1 or SUMO-2/3 isoforms [19] and it was further shown that individual SUMO isoforms have different dynamic properties *in vivo* [20]. However, disruption of both *sumo-1* alleles in mouse did not lead to observable developmental defects, which implies that SUMO-2/3 can substitute function of SUMO-1 [21]. SUMO-2/3 can form polymeric chains through lysine residue Lys11 located in the SUMOylation motif of their flexible N-termini [22,23]. The formation of SUMO-1 chains via unknown non-consensus sites was also observed both *in vitro* and *in vivo* [14,10], but it is unclear at this point whether they are of any biological relevance. It was further reported that SUMO-2/3 conjugates accumulated under different stress conditions [24] and that part of these conjugates represented SUMO-2/3 chains [25]. Moreover, ubiquitin associated SUMO-2/3 conjugates accumulated under proteasomal inhibition [26] and similar results were observed with Smt3 in yeast [27]. SUMO-targeted ubiquitin ligases RNF4 and Slx5/Slx8 were identified in vertebrates and yeast, respectively, as the main linking factor between SUMOylation and

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* Corresponding author. Fax: +420 220445167.
E-mail address: knejzliz@vscht.cz (Z. Knejzlík).

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ubiquitylation [28,27]. It has been shown that RNF4 is able to bind and ubiquitylate SUMO-2 chains *in vitro* and that siRNA-based lowering/depletion of RNF4 level leads to increased stability of SUMO-1 and SUMO-2 conjugates [29]. Disruption of genes for RNF4 orthologues in yeast also resulted in accumulation of Smt3 conjugates [28,30].

Although accumulation of SUMO-2/3 conjugates was described under different stress conditions and the set of SUMO-2/3 targets under heat shock or MG132 treatment has been determined [25,26], the mechanism of target selection as well as the significance of this SUMO-2/3 conjugate accumulation remain to be determined. For this reason, we were interested whether accumulation of SUMO-2/3 conjugates under heat shock or MG132 treatment was somehow linked with the stress response on the transcription and translation level. However, our data indicated that SUMO-2/3 conjugate accumulation was rather linked with the global *de novo* protein synthesis under these conditions. Here we show that SUMO-2/3 conjugates accumulating both under heat shock and MG132 treatment come mainly from new protein synthesis.

2. Materials and methods

2.1. Plasmid constructs

Plasmids encoding cMyc-SUMO-1 (pCMV-cMyc-SUMO1), HA-SUMO-3 (pCMV-HA-SUMO3) and non-conjugable HA-SUMO-3 ΔG (pCMV-HA-SUMO3 ΔG) were prepared as follows: SUMO-1 and SUMO-3 coding sequences were isolated by RT-PCR from total RNA of HeLa cells using primers S1+ (cgGAATTCtgtctgaccaggaggcaaaaccttc) and S1− (ttttCTCGAGttaacccccgtttgttcctgataaact) for SUMO1 and S3+ (cgGAATTCtgtccgaggagaagcccaaggaggg) and S3− (ttttCTCGAGttaacctcccgtctgctgctggaacacgt) for SUMO3. To construct pCMV-cMyc-SUMO1 and pCMV-HA-SUMO3 plasmids, the SUMO-1 and SUMO-3 coding sequences were inserted into the *Eco*RI and *Xho*I sites of pCMV-cMyc and pCMV-HA plasmids (Clontech), respectively. Plasmid pCMV-HA-SUMO3ΔG encodes SUMO-3 with deletion of Gly 92. This SUMO-3ΔG sequence was obtained from pCMV-HA-SUMO3 plasmid by PCR, using S3+ and S3ΔG− (cgcGGATCCttatccgtctgctgctggaacacgtcg) primers and then inserted into *Eco*RI and *Bam*HI sites of pCMV-HA plasmid. Plasmid pCMV-HA-SUMO3(ΔN) encodes SUMO-3 with deletion of the first 10 N-terminal amino acid residues and with Lys11 to Ala11 substitution. This SUMO-3ΔN sequence was obtained from pCMV-HA-SUMO3 plasmid by PCR, using S3 ΔN+ (CGGAATTCaaGCAacagagaatgaccacatcaacctgaagg) and S3− primers and then inserted into *Eco*RI and *Xho*I sites of pCMV-HA plasmid. All obtained DNA constructs were verified by sequencing.

2.2. Cell cultures and transient transfection

HEK 293T, HeLa and U2OS cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and vitamins (both from Invitrogen) and penicillin/streptomycin/amphotericin B solution (Sigma-Aldrich). Four hours prior to transfection, cells were plated on 35 mm tissue culture dish to 50% density and transient transfection was carried out using polyethyleneimine (PEI; Polysciences) as follows: 4 μg of plasmid DNA and 12 μl of PEI solution (1 μg/μl PEI in ddH_2O) were mixed separately with 100 μl FBS-free DMEM. After 15 min, solutions were mixed together and transferred dropwise to the culture dish. Expression of transfected genes was analyzed 12 h post-transfection by Western blot.

2.3. Western blot analysis

Cells on 35 mm tissue culture dish were directly lysed in 150 μl of 2× concentrated Laemmli buffer (100 mM Tris, pH 6.8, 8% SDS, 24% glycerol, 0.02% Coomassie blue G-250, 200 mM dithiothreitol), boiled for 5 min and sonicated to lower sample viscosity. Cell lysates were separated by 9.6% Tris-tricine SDS-PAGE and electroblotted to a nitrocellulose membrane. Membrane was incubated in blocking solution (5% fat-free milk, 0.1% Tween-20 in PBS) for 1 h. Endogenous SUMO-2/3 was detected using polyclonal anti-SUMO-2 antibody (Invitrogen) in dilution 1:750. Myc and HA fused proteins were detected using relevant epitope tag specific, mouse monoclonal antibodies (both purchased form Sigma-Aldrich), in dilution 1:5000. Protein level normalization was carried out by rabbit polyclonal anti-GAPDH antibody (Sigma-Aldrich), used in dilution 1:10,000. Secondary anti-mouse and anti-rabbit IgG specific HRP-conjugated antibodies (Promega) were used in dilution 1:4000. Immunoblots were visualized by Super Signal West Femto chemiluminescent substrate (Pierce) according to the manufacturer's instructions. The quantification of immunoblots was carried out using TotalLab TL100 software (Nonlinear USA Inc., NC, USA).

2.4. Inhibition of translation, proteasome inhibition, heat shock and inhibition of Hsp 90

To inhibit translation, cells were treated with 30 μg/ml cycloheximide or 100 μg/ml puromycin for 20 min. Proteasomal activity was inhibited by 20 μM MG132. All inhibitors were purchased from Sigma-Aldrich. Heat shock was performed by incubation of the cells for 45 min in a CO_2 incubator preheated to 42 °C. In case of combination of inhibitor treatment and heat shock, cells were first subjected to either translational or proteasomal inhibitor for 20 min. Subsequently and without removal of the particular inhibitor, each cell sample was then subjected to an additional 45 min of heat shock. In case of combination of translational and proteasomal inhibitor treatment, cells were first subjected to translational inhibition for 20 min. Subsequently and without removal of the translational inhibitor, each cell sample was then exposed to proteasomal inhibitor MG132 for 80 min. To inhibit Hsp 90 activity, cells were treated by 20 μM 17-AAG (Selleck) for 40 min.

2.5. Induction and monitoring of synthesis of truncated proteins

To test the ability of different concentrations of puromycin to induce truncated protein synthesis, HEK 293T cells on 35 mm Petri dish were washed twice by PBS and cultivated in FBS-free DMEM without methionine/cysteine (Sigma-Aldrich) in presence of puromycin in range from 0.5 to 100 μg/ml. After 20 min, [^{35}S]-methionine/cysteine (TRAN^{35}S-LABEL™; MP Biomedicals) was added to final specific activity 50 μCi/ml. After 20 min incubation, the labeling medium was discarded and cells were directly lysed in 500 μl of 2× concentrated Laemmli buffer. Total lysates were boiled for 10 min and analyzed by Tris-tricine SDS-PAGE. Radiolabeled translation products were visualized by phosphorimaging. To induce synthesis of truncated proteins in our experiments, the cells were treated with 10 μg/ml puromycin for 20 min.

2.6. SUMO deconjugation

For SUMO deconjugation from intracellular targets, HEK 293T cells were pelleted and the cell pellet was resuspended in 100 μl of native lysis buffer (50 mM TrisHCl, 150 mM NaCl, 0,4% NP-40, 1 mM DTT, 1 mM EDTA, 20 μM MG132, 20 μg/ml Leupeptin, 1 mM PMSF, pH 8), alternatively containing *S. cerevisiae* Ulp1 (Invitrogen). The lysates were incubated for 2 h at room temperature.

2.7. Determination of SUMOylation of newly synthesized proteins

HEK 293T cells were transfected by plasmid pCMV-HA-SUMO-3 at 50% density on 60 mm Petri dish. After 12 h cells were washed two-times by DMEM without methionine/cysteine and radiolabeled by

[^{35}S]-methionine/cysteine (100 μCi/ml) (TRAN^{35}S-LABEL™; MP Biomedicals) for 60 or 120 min at conditions described in individual experiments. Cells were released by 1 ml of the original cultivation medium and the resulting cell suspension was equally divided into two parts (designated here as portion A and B). Cells were pelleted at 1000×*g* for 1 min and the cell pellet was treated as follows. *Portion A* (deconjugation of HA-SUMO-3): HA-SUMO-3 was deconjugated as described in *2.6*, SDS was then added to final concentration of 2%. Lysates were boiled for 10 min, intensively sonicated and diluted by 900 μl of dilution buffer (10 mM Tris.HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, pH 8). *Portion B* (isolation of HA-SUMO-3 conjugates): The cell pellet was resuspended in 100 μl of denaturation lysis buffer (10 mM Tris.HCl, 150 mM NaCl, pH8, 2% SDS, 10 mM NEM, 5× conc. inhibitor cocktail (Complete, Roche), 1 mM PMSF), boiled for 10 min, intensively sonicated and diluted by 900 μl of dilution buffer. Following operations were identical for both A and B lysates. Diluted lysates were incubated at 4 °C for 30 min on a rotator, centrifuged (20,000×*g*, 30 min, 20 °C) and supernatant was harvested. 40 μl of 50% suspension of anti-HA agarose (Sigma) was added to the supernatant and incubated at 4 °C for 3 h on a rotator. The resin was washed four-times by washing buffer (10 mM Tris.HCl, 1 M NaCl, 1% NP-40, 1 mM EDTA, pH 8). The resin was then resuspended in 5 ml of liquid scintillation cocktail and radioactivity of samples was measured by liquid scintillation detector (Beckman).

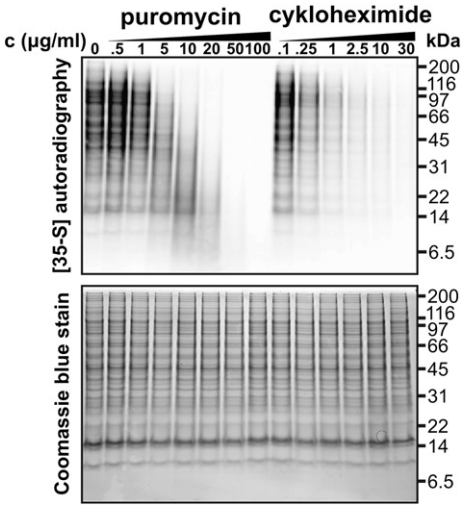

Fig. 1. Effect of puromycin or cycloheximide treatment on protein synthesis. HEK 293T cells were treated with different concentrations of puromycin or cycloheximide for 20 min and then subjected to the [^{35}S]-methionine radiolabeling of translated products for 20 min. Total cell lysates were analyzed by SDS-PAGE and radiolabeled translation products were visualized by phosphorimaging (*upper panel*). Coomassie blue staining was used as a control of total protein levels (*lower panel*).

3. Results

3.1. Effect of translational inhibitors on accumulation of SUMO-2/3 conjugates under heat shock and under proteasomal inhibition

It has been previously shown that in contrast to SUMO-1, the level of SUMO-2/3 conjugates is increased in response to various types of stress including heat shock [24] and, furthermore, the set of SUMO-2/3 targets under heat shock has been determined [25]. We followed these studies and investigated effect of translational inhibitors, cycloheximide and puromycin, on accumulation of SUMO-2/3 conjugates under the heat shock. Translation inhibition should abolish the transcriptional heat shock response by inactivation of *de novo* protein synthesis. To avoid possible detrimental side-effects of translational inhibitors, we first tested the impact of different concentrations of the inhibitors on protein synthesis over a short time treatment. HEK 293T cells were treated for 20 min with puromycin at concentration range 0.5–100 μg/ml or with cycloheximide at concentration range 0.1–30 μg/ml with consequent 20 min [^{35}S]-methionine radiolabeling. The molecular mass and amounts of the synthesized proteins were determined by SDS-PAGE followed by autoradiography (Fig. 1). Puromycin at concentration 100 μg/ml and cycloheximide at concentration 30 μg/ml totally inhibited protein synthesis. However, in contrast to cycloheximide, puromycin at lower concentrations (5, 10 and 20 μg/ml) caused visible shortening of newly synthesized proteins (Fig. 1).

To totally block protein synthesis, HEK 293T, U2OS and HeLa cells were treated with cycloheximide (30 μg/ml) or puromycin (100 μg/ml) and the cells were then subjected to the heat shock. Total cell lysates were analyzed by Western blot for the content of SUMO-2/3 conjugates. U2OS and HEK 293T cells untreated by translational inhibitors accumulated SUMO-2/3 conjugates under heat shock, which correlates with the lower level of free (unconjugated) SUMO-2/3 when compared to the control cells cultivated at 37 °C (Fig. 2A and C, compare lanes 1 and 2). However, pretreatment of U2OS and HEK 293T cells by puromycin and cycloheximide was linked with block of SUMO-2/3 conjugates accumulation under heat shock. This observation was also in correlation with the level of free SUMO-2/3, which was comparable to the cells, both untreated and treated by translational inhibitors, cultivated at 37 °C (Fig. 2A and C, compare lanes 3 and 4, 5 and 6, free SUMO-2/3). In HeLa cells, heat shock induced accumulation of SUMO-2/3 conjugates under our experimental conditions was not observed (Fig. 2B, compare lines 1 and 2), therefore the effect of translation inhibitors could not be determined.

Since accumulation of SUMO-2/3 conjugates has been reported also under proteasomal inhibition, both in yeast and mammalian cells [26,27], we investigated effect of the translational inhibitors on accumulation of SUMO-2/3 conjugates under MG132 treatment. HEK 293T, U2OS and HeLa cells were treated with translational inhibitor and subsequently with proteasomal inhibitor MG132. When cells were treated by MG132 alone, all tested cell lines showed accumulation of SUMO-2/3 conjugates (Fig. 2A–C, compare lanes 7 and 8). In case of pretreatment by translational inhibitors, the MG132-induced accumulation of SUMO-2/3 conjugates was blocked in all three tested cell lines (Fig. 2A–C, compare lanes 9 or 10 with lane 8).

The intracellular level of SUMO-2/3 conjugates is a result of equilibrium between their formation on one side and deconjugation and/or degradation on the other side. For this reason, we examined effect of cycloheximide treatment on SUMO-2/3 conjugates accumulation during heat shock in combination with proteasomal inhibition in HEK 293T cells (Fig. 3). As already shown above, inhibition of protein synthesis blocked both the heat shock and MG132 induced SUMO-2/3 conjugates accumulation (Fig. 3, compare lane 3 with 4 and lane 5 with 7, respectively; Fig. 4A, compare conditions 1 with 2 and 3, and next, 7 with 8 and 9, respectively). However, accumulation of SUMO-2/3 conjugates under heat shock and translational inhibition was restored by MG132 treatment to the level comparable with control untreated cells under the heat shock (Fig. 3, compare lane 4 with 8 and next lane 2 with 8, Fig. 4A, compare conditions 2 with 4 and 3 with 5). That suggests that inhibition of translation did not abolish SUMOylation itself. Furthermore, the inhibition of translation did not lead to the decrease of the intracellular SUMO level (Supplementary Fig. 1) but rather it somehow influenced the level of the SUMOylation targets.

3.2. Newly synthesized proteins as targets of SUMOylation

In the above-described experiments, we used puromycin in concentration completely abolishing translation (100 μg/ml). Since the treatment with low concentration of puromycin (5, 10 and

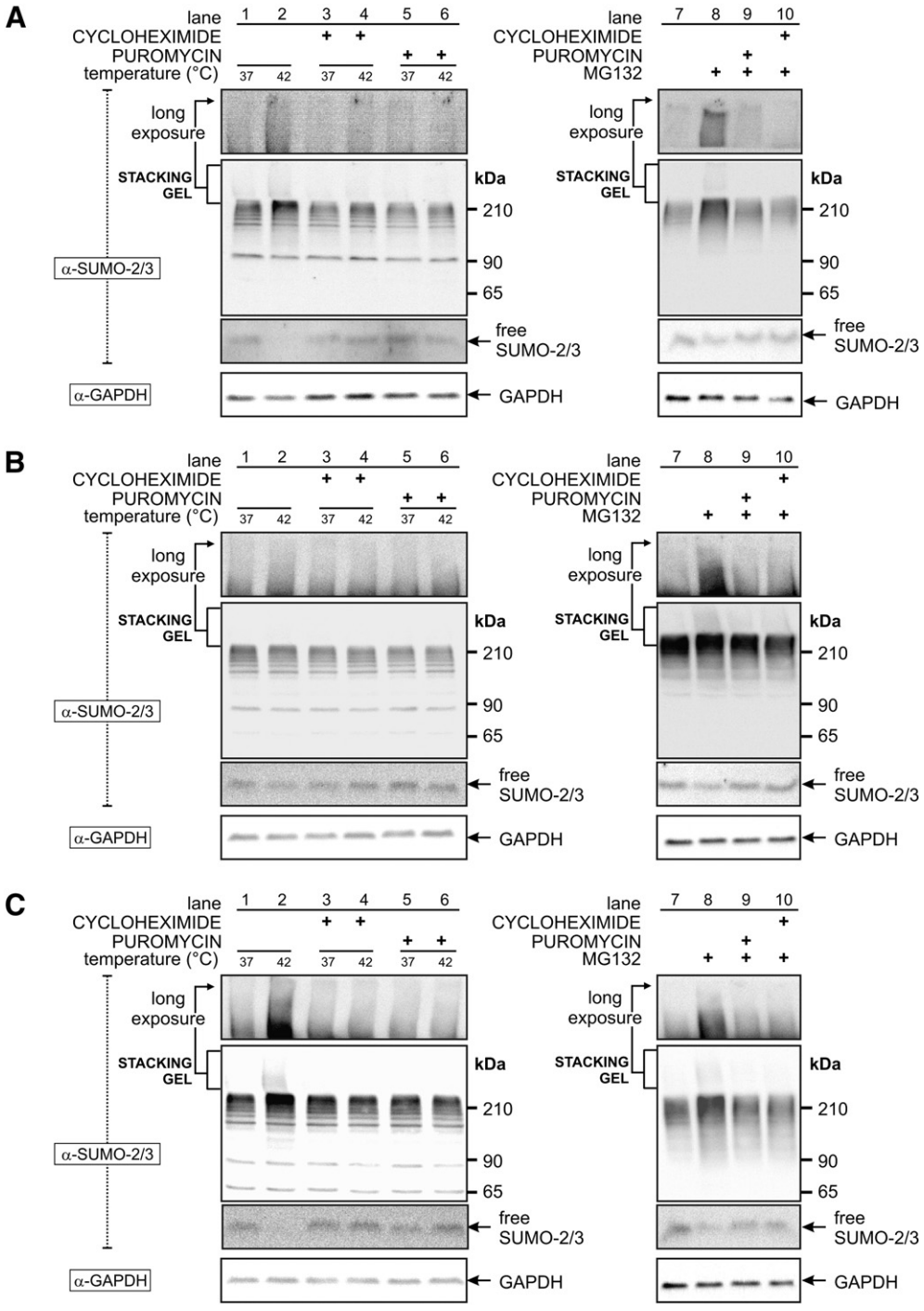

Fig. 2. Effect of translational inhibitors on accumulation of SUMO-2/3 conjugates. Total cell lysates were analyzed by Western blot followed by immunodetection using anti-SUMO-2/3 antibody. *A. Left panel:* U2OS cells were treated with translation inhibitors, 30 μg/ml cycloheximide or 100 μg/ml puromycin, for 20 min with consequent 45 min heat shock (42 °C). Inhibition of translation prevented the heat shock induced accumulation of SUMO-2/3 conjugates (lanes 4 and 6). *Right panel*: U2OS cells were treated with translation inhibitors, 30 μg/ml cycloheximide or 100 μg/ml puromycin, for 20 min with consequent 80 min MG132 treatment. Inhibition of translation prevented the MG132 induced accumulation of SUMO-2/3 conjugates (lanes 9 and 10). *B.* Same as in *A.*, but HeLa cells were used. In case of heat shock, HeLa cells did not accumulate SUMO-2/3 conjugates under our experimental conditions, so the effect of translation inhibitors could not be determined. *C.* Same as in *A.*, but HEK 293T cells were used.

20 μg/ml) allows us to generate incompletely translated proteins (peptides) (Fig. 1), we decided to investigate the effect of synthesis of truncated proteins on SUMO-2/3 conjugates accumulation. To induce formation of defective truncated proteins in our experiments we chose puromycin concentration of 10 μg/ml (Fig. 1).

HEK 293T cells were treated with puromycin (10 μg/ml or 100 μg/ml) for 20 min and subsequently the proteasome inhibitor MG132 was added for next 20 or 80 min. The cell lysates were analyzed by Western blot using antibody against SUMO-2/3 (Fig. 4B). Puromycin at concentration 100 μg/ml prevented the MG132-induced accumulation of SUMO-2/3 conjugates (Fig. 4B, compare lanes 4 and 12, Fig. 4A, compare conditions 7 and 8). On the other hand, treatment with low concentration of puromycin (10 μg/ml) in combination with MG132 resulted in accumulation of not only high molecular mass SUMOylated species, but of SUMOylated species of lower molecular mass (approx. from 35 kDa) as well (Fig. 4B, lane 8, Fig. 4A, condition 10). Since the puromycin treatment at concentration 10 μg/ml led to production of defective

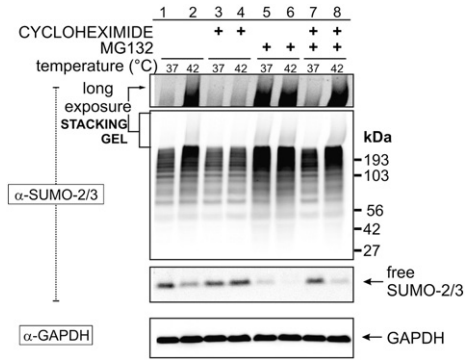

Fig. 3. Effect of cycloheximide and MG132 on accumulation of SUMO-2/3 conjugates under heat shock. HEK 293T cells were pre-treated with 30 μg/ml cycloheximide for 20 min, then 20 μM MG132 was added for another 20 min and consequently the cells were subjected to heat shock (42 °C) for 45 min. MG132 alone induced SUMO-2/3 conjugates accumulation both at 37 °C and at 42 °C (lanes 7 and 8). At 37 °C, the MG132 induced SUMO-2/3 conjugates were considerably reduced when translation was first inhibited by cycloheximide (lane 9). However, at 42 °C and under MG132 treatment the accumulation of SUMO-2/3 conjugates was not prevented by translation inhibition (lane 10).

truncated proteins with molecular mass interval approximately between 6 and 45 kDa (Fig. 1, puromycin 10 μg/ml), we suppose that the low molecular mass SUMOylated species represent some of these truncated proteins modified by SUMO-2/3. Furthermore, HA-SUMO-3 and Myc-SUMO-1 were co-expressed and the effect of puromycin on their conjugation was tested (Fig. 5A). The accumulation of HA-SUMO-3 conjugates under proteasomal inhibition after the puromycin (10 μg/ml) treatment showed similar pattern as in case of endogenous SUMO-2/3 (Fig. 5A, lower panel). On the other hand, no visible change in conjugation of Myc-SUMO-1 was observed in any case of puromycin or MG132 treatment, or their combination (Fig. 5A, upper panel). Formation of the high molecular mass SUMO-2/3 or HA-SUMO-3 conjugates under the puromycin treatment (10 μg/ml) could originate from polySUMOylation. This possibility is supported by the decrease of molecular mass of the HA-SUMO-3ΔN mutant conjugates, lacking among others the Lys11 residue responsible for SUMO-2/3 chain formation (Fig. 5B).

Under the puromycin treatment (10 μg/ml), formation of ubiquitylated SUMO-2/3 or HA-SUMO-3 truncated products cannot be excluded. However, in the case of over-expression of non-conjugable HA-SUMO-3ΔG mutant, puromycin treatment in combination with MG132 did not result in formation of any other species recognized by anti-HA antibody than free SUMO-3ΔG (Supplementary Fig. 2). This supports our assumption that the low molecular mass species represent truncated proteins modified by either SUMO-2/3 or HA-SUMO-3.

The blocked accumulation of SUMO-2/3 conjugates under MG132 or 42 °C treatment when translation is inhibited, and on the other hand, the accumulation of SUMO-2/3 conjugates when truncated protein synthesis is induced, suggests that some of the newly synthesized proteins may be targets for SUMOylation. This possibility was confirmed by immunoprecipitation of HA-SUMO-3 conjugates from radiolabeled HEK 293T cells. To avoid false positive results caused by newly synthesized and therefore radiolabeled, HA-SUMO-3, each sample was equally divided and HA-SUMO-3 in one aliquot was deconjugated *in vitro* by endogenous SUMO proteases in combination with added Ulp1 (Supplementary Fig. 3) prior to immunoprecipitation of HA-SUMO-3 under denaturing conditions. Ratio of radioactivity (cpm values) of conjugated/deconjugated HA-SUMO-3 imunoprecipitates from unstressed HEK 293T cells was near to 1:1, indicating that HA-SUMO-3 was not conjugated to newly synthesized proteins (Fig. 6). On the other hand, the cpm ratio was in interval from 1.4 to 1.6 for cells cultivated in presence of MG132 (120 min) or MG132 and 10 μg/ml puromycin (120 min) or under 42 °C (60 min). This

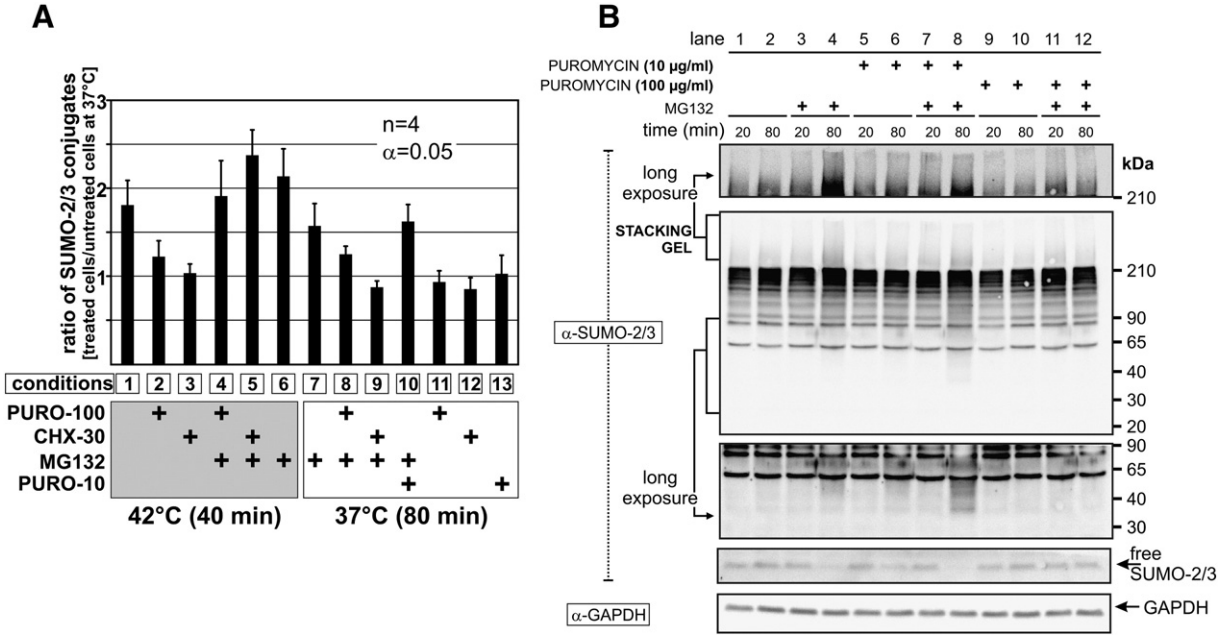

Fig. 4. SUMOylation of newly synthesized truncated proteins. *A.* Quantification of SUMO-2/3 conjugates content under different conditions in HEK 293T cells. *y* axis represents ratio of content of SUMO-2/3 conjugates in cells at appropriate conditions to SUMO-2/3 conjugates in control untreated cells cultivated at 37 °C. Treatments were carried out as described in Materials and methods. The immunoblots were analyzed by TotalLab TL100 software. PURO-100 or PURO-10 — puromycin 100 μg/ml or 10 μg/ml, CHX-30 — cycloheximide 30 μg/ml. Error bars represent confidence for $\alpha = 0.05$ and four independent experiments ($n = 4$). *B.* HEK 293T cells were treated with puromycin for 20 min with consequent MG132 treatment for next 20 or 80 min. Total cell lysates were analyzed by Western blot using anti-SUMO-2/3 antibody. After the addition of low concentration of puromycin (10 μg/ml) in combination with MG132, cells accumulated not only high molecular mass SUMOylated species but also SUMOylated species of lower molecular mass (cca from 35 kDa, see lane 8). These low molecular mass SUMOylated species were also slightly visible after prolonged exposure of control cells to MG132 (lane 4), but not in the case when translation was first totally inhibited by 100 μg/ml puromycin with consequent exposition to the proteasome inhibition (lane 12).

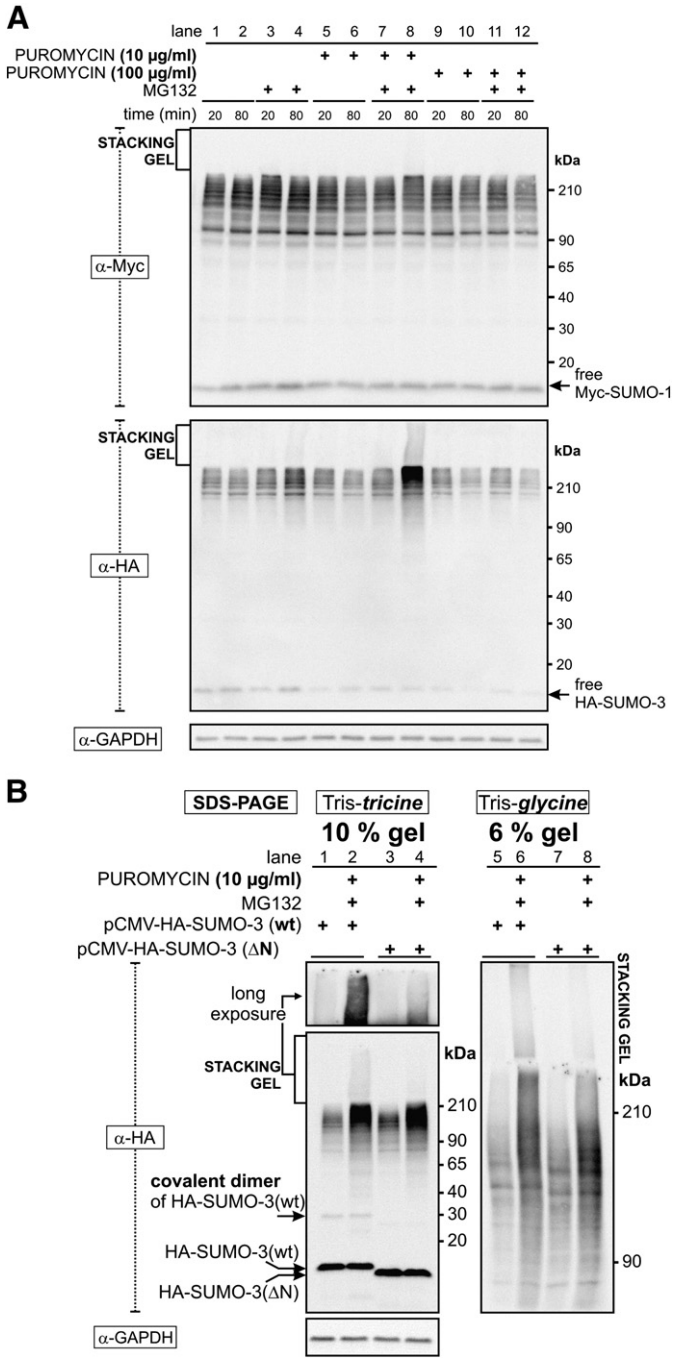

Fig. 5. SUMOylation of truncated proteins preferentially by HA-SUMO-3. Total cell lysates were analyzed by Western blot followed by immunodetection using anti-HA or anti-Myc antibody. *A.* HEK 293T cells were co-transfected with pCMV-HA-SUMO-3 and pCMV-Myc-SUMO-1 and then treated as described in legend of Fig. 3B. Both low and high molecular mass conjugates accumulated after induction of defective short protein synthesis and proteasome inhibition in case of HA-SUMO-3 (*lower panel*, lane 8), but not in case of Myc-SUMO-1 (*upper panel*, lane 8). *B.* HEK 293T cells were transfected with pCMV-HA-SUMO-3 or pCMV-HA-SUMO-3ΔN, encoding HA-SUMO-3 mutant defective in Lys11 chain formation. Transfected cells were treated with 10 μg/ml puromycin for 20 min with consequent MG132 treatment for 80 min and total cell lysates were analyzed by 10% Tris-tricine (*left panel*) or 6% Tris-glycine (*right panel*) SDS-PAGE followed by Western blot analysis. In both cases, induced accumulation of SUMO conjugates was observed under the treatment, however, molecular mass of SUMO conjugates was decreased in case of HA-SUMO-3ΔN mutant.

higher cpm ratio indicates that some of the newly synthesized proteins were covalently associated with HA-SUMO-3 under these conditions.

The fact that the truncated proteins synthesized after puromycin (10 μg/ml) treatment are highly unstable *in vivo* and their stability depends on proteasomal activity (Supplementary Fig. 4) suggests that their folding could be compromised due to premature termination of translation. In this context we examined effect of 17-AAG, the inhibitor of Hsp 90 activity [31,32], on SUMOylation (Fig. 7). Treatment of HEK293T cells by combination of 17-AAG and MG132 for 40 min resulted in accumulation of SUMO-2/3 conjugates, which was not observed when only MG132 was used or when translation was first inhibited by cycloheximide.

4. Discussion

In this study, we showed that both the heat shock and MG132 induced accumulation of SUMO-2/3 conjugates can be blocked by inhibition of translation in HEK 293T and U2OS cells. Although the study is based mainly on the use of chemical inhibitors we firmly believe that our results are significant. To eliminate the possibility of side effects of the translation inhibitors we used two mechanistically different translational inhibitors, puromycin and cycloheximide. In case of puromycin, the puromycyl peptides are released from ribosome, ribosomal subunits dissociate from mRNA and they are ready for the new initiation of translation [33]. Cycloheximide, on the other hand, blocks ribosomal peptidyltransferase activity, which results in polysomes stabilization [34,35]. Moreover, in this study the inhibitors were used for short time periods to avoid detrimental biological side effects.

The accumulation of SUMO-2/3 conjugates under different stress conditions may be caused by various events. For example, activity of proteasomes or SUMO proteases could be decreased, activity of SUMOylation machinery could be increased, new types of SUMO-2/3 targets could be generated or combination of these events might occur. Previously, it was shown that treatment with MG132 or other types of proteasomal inhibitors increase the level SUMO-2/3 conjugates *in vivo* [26,27], which suggest that proteasomes could play an important role in the SUMO-2/3 conjugates stability. Our data showed that the MG132-induced accumulation of endogenous SUMO-2/3 conjugates was blocked when translation was first abolished by treatment with cycloheximide or puromycin, in all three tested cell lines — HeLa, U2OS and HEK 293T (Fig. 2 A–C, lines 7–10). It is necessary to note that accumulation of HA-SUMO-3 conjugates in HEK 293T under proteasomal inhibition was also abolished by block of translation (Fig. 5A, lower panel, compare line 4 and 12). Since both these mechanistically different translational inhibitors have similar negative impact on MG132-induced accumulation of endogenous SUMO-2/3 conjugates, we suggest, that the SUMOylation is directly linked with proteosynthetic activity of ribosomes, namely, there is relation between accumulation of SUMO-2/3 conjugates and production of newly synthesized proteins during MG132 treatment. This idea is supported by the results of the experiment in which combination of MG132 with 10 μg/ml puromycin was used. Puromycin at this concentration did not block translation completely, but it caused production of truncated proteins with mobility corresponding up to ~45 kDa (Fig. 1). Puromycin treatment at this concentration was accompanied by accumulation of endogenous SUMO-2/3 conjugates of lower molecular mass (Fig. 4B, lane 8). Furthermore, when HA-SUMO-3 and Myc-SUMO-1 were co-expressed, only low molecular mass conjugates of HA-SUMO-3 did accumulate, but not those of Myc-SUMO-1 (Fig. 5A, lane 8). We suggest that these low molecular mass SUMO-2/3 or HA-SUMO-3 conjugates represent part of the newly synthesized truncated proteins, namely puromycyl peptides, modified by SUMO-2/3 or HA-SUMO-3 and that their stability depends on proteolytic activity of proteasomes. It was previously reported that puromycyl peptides are rapidly degraded *in vivo* [36,37] and we suggest that a part of them is modified by SUMO-2/3 before degradation. However, puromycin at concentration 10 μg/

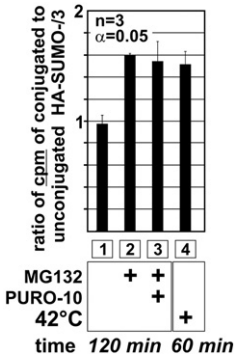

Fig. 6. Conjugation of HA-SUMO-3 to newly synthesized proteins. HEK 293T cells over-expressing HA-SUMO-3 were radiolabeled using [^{35}S]-methionine for 120 or 60 min at indicated conditions (1–37 °C; 2–37 °C, 10 μM MG132; 3–37 °C, 10 μM MG132, 10 μg/ml puromycin; 4–42 °C) and both conjugated and deconjugated HA-SUMO under indicated conditions were immunoprecipitated in parallel as described in Section 2.7. Data are presented as a ratio of cpm of conjugated to deconjugated HA-SUMO-3 in the immunoprecipitate. Error bars represent confidence for $\alpha = 0.05$ and three independent experiments ($n = 3$).

ml in combination with MG132 also induced formation of high molecular mass SUMO conjugates as well, especially when HA-SUMO-3 was over-expressed (Fig. 5A, lower panel, lane 8). These might represent truncated proteins with multiple (poly)SUMO-2/3 modifications (Fig. 5B), additional ubiquitylation of which cannot be excluded [22,23,25–27]. The low molecular mass SUMOylated species may be also, but in a lower content, detected in the MG132 treated cells (Fig. 4B, line 4), but not when translation was first completely inhibited by 100 μg/ml puromycin and when the proteasomes were consequently inhibited (Fig. 4B, line 12). We suggest that these low molecular mass SUMO conjugates result from naturally occurring aberrant translational products and that they accumulate under proteasomal inhibition.

Cycloheximide and puromycin also blocked the accumulation of heat-induced SUMO-2/3 conjugates in U2OS and HEK 293T cells (Fig. 2A and C, lanes 1–6). However, since the accumulation of SUMO-2/3 conjugates under heat shock and translation inhibition was restored by MG132 treatment (Fig. 3, compare line 4 with 8), we suggest that the inhibition of translation during the heat shock do not abolish SUMOylation itself. Rather, it affected the level of SUMOylation targets and most of the newly formed SUMO-2/3 conjugates under the heat shock, when translation is inhibited, are degraded by proteasomes. From this perspective, it looks as though the SUMO-2/3 conjugates accumulated during heat shock comprise both the SUMO-2/3 modified newly synthesized proteins and the SUMO-2/3 modified proteins synthesized before the heat shock. On the other hand, especially newly synthesized proteins seem to be SUMOylated under MG132 treatment (Fig. 3, compare lanes 3, 5 and 7). Covalent HA-SUMO-3 association with subset of newly translated proteins (Fig. 6) documents that there is a direct connection between HA-SUMO-3 conjugation and new protein synthesis under MG132 or MG132/puromycin (10 μg/ml) treatment or under heat shock.

The dependence of SUMO-2/3 conjugates stability on proteasomal activity both at 37 °C and 42 °C generates a question, which types of proteins are selected as targets. Many SUMOylated proteins have been already identified both under normal and stress conditions [9,25]. However, biological significance of the stress induced SUMOylation is not yet clear. It is well known that natural transcriptional/translational errors lead to production of unfolded or misfolded proteins and that their load could be increased under different stress stimuli. This can happen primarily due to the direct impact of stress factors on protein folding and secondary due to the saturation of a chaperone system. It is possible that just a part of these unfolded/misfolded proteins is SUMOylated under stress conditions. This is supported by the experiments with puromycin (10 μg/ml) treatment, which resulted in formation of low molecular mass SUMO-2/3 conjugates, representing part of the newly synthesized truncated proteins modified by SUMO-2/3, folding of which could be compromised due to premature termination of their translation. Our assumption about incorrect folding of a substantial subset of the truncated proteins is supported by their rapid degradation by proteasomes (Supplementary Fig. 4). Furthermore, we showed a correlation between inhibition of activity of Hsp 90 by 17-AAG, the chaperone included in the last step of folding machinery [38,39], and increased accumulation of SUMO-2/3 conjugates (Fig. 7). The fact that inhibition of translation abolished the accumulation of SUMO-2/3 conjugates under the 17-AAG/MG132 treatment suggests that the fold of the newly synthesized proteins could be a critical parameter for SUMO-2/3 target selection. An example of the relationship between protein misfolding and SUMOylation has already been reported for PML protein [40]. In this case, binding of As(III) ion to PML zinc finger led to conformational change and aggregation of PML, which then efficiently interacted with Ubc9 and was polySUMO-2ylated *in vitro*. Another example is SUMOylation of a yeast transcription factor Mot1, which is targeted for Smt3 mediated degradation due to a temperature sensitive mutation or due to the incorporation of canavanine, an arginine analog [41]. SUMOylation of other proteins increased upon canavanine treatment as

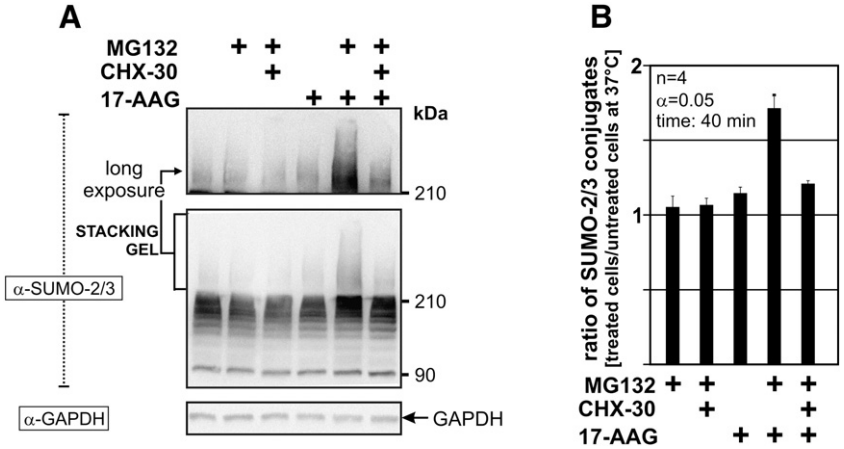

Fig. 7. Effect of 17-AAG on accumulation of SUMO-2/3 conjugates. HEK 293T cells were pre-treated with 30 μg/ml cycloheximide for 20 min, then 20 μM MG132 was added with or without 20 μM 17-AAG for another 40 min at 37 °C. *A.* Immunochemical detection of SUMO-2/3 conjugates. *B.* Quantification of SUMO-2/3 conjugates. Error bars represent confidence for $\alpha = 0.05$ and four independent experiments ($n = 4$). After 40 min, MG132 alone did not induce SUMO-2/3 conjugates accumulation. However, their accumulation was observed when MG132/17-AAG was used.

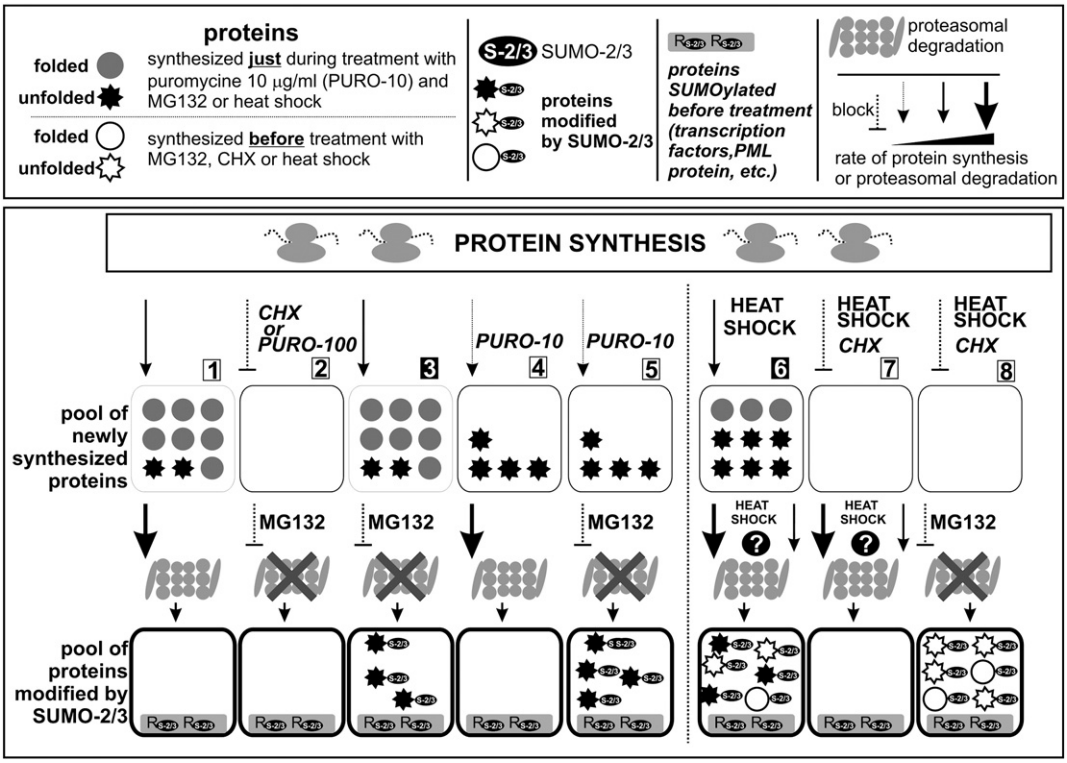

Fig. 8. Scheme illustrating suggested relation between translation and accumulation of SUMO-2/3 conjugates. Under normal conditions (1), the detected SUMO-2/3 conjugates represent mainly various modified nuclear factors (*e.g.* PML protein). If translation is blocked by cycloheximide (CHX) or puromycin 100 μg/ml (PURO-100) under MG132 treatment (2), accumulation of SUMO-2/3 conjugates is not observed. When translation is not blocked during MG132 treatment (3), the SUMO-2/3 conjugates accumulate. When synthesis of short aberrant proteins is induced by puromycin 10 μg/ml (4), the SUMO-2/3 conjugates do not accumulate, however, they are observed when proteasomal activity is blocked (5). Part of these conjugates is of lower molecular mass compared to case (3). These data indicate that during MG132 treatment mainly unfolded/misfolded newly synthesized proteins modified by SUMO-2/3 do accumulate. Under heat shock, the SUMO-2/3 conjugates accumulate (6), but not when translation is blocked (7). However, the accumulation of SUMO-2/3 conjugates can be restored by proteasomal inhibition (8). Taken together, the SUMO-2/3 conjugates accumulated under heat shock comprise both the SUMO-2/3 modified newly synthesized proteins and the SUMO-2/3 modified proteins synthesized before the heat shock.

well, and at least one other protein, Spt20, was reported to be degraded upon canavanine treatment [41].

These reported findings together with our data support our hypothesis that a part of the newly synthesized unfolded/misfolded proteins could be SUMOylated and that SUMOylation might represent a quality control mark of protein folding. However, the "added value" of SUMOylation is so far unclear, especially when ubiquitylation has been shown to be directly linked with co-translational control of protein folding [42–45].

5. Conclusion

We showed that conjugates of endogenous SUMO-2/3 accumulating under heat shock or MG132 treatment largely represent modified proteins generated from new protein synthesis. Next, we propose that these SUMOylated targets could be unfolded/misfolded proteins and stability of these conjugates depends on proteasomal activity (Fig. 8).

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2012.01.010.

Authors comment

During the revision of this manuscript, Tatham et al. independently reported some similar observations [46]. Using different experimental approach, they have shown that, among others, accumulation of SUMO-2/3 conjugates under proteasomal inhibition depends on protein synthesis and they have suggested a role for SUMO in protein quality control. These observations and conclusions are in agreement with our findings.

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