Supporting Online Material for

Protein Synthesis upon Acute Nutrient Restriction Relies on Proteasome Function
Ramunas M. Vabulas* and F. Ulrich Hartl*

*To whom correspondence should be addressed. E-mail: vabulas@biochem.mpg.de (R.M.V.); uhartl@biochem.mpg.de (F.U.H.)

DOI: 10.1126/science.1121925

This PDF file includes:

Materials and Methods
Figs. S1 to S4
Table S1
Supporting Online Material

Materials and Methods

Reagents and antibodies

MG132 was from Biomol (Playmouth Meeting, PA), clasto-lactacystin-β-lactone, epoxomicin, bafilomicin A1 and bestatin methyl ester from Calbiochem (San Diego, CA), PBS was from Gibco-Invitrogen (Carlsbad, CA), butanol and acetic acid from Merck (Darmstadt, Germany) and L-leucine, L-phenylalanine, L-methionine, DMSO, cycloheximide, chloroquine, 3-methyladenine, L-azetidine 2-carboxylic acid, and trichloroacetic acid (TCA) were from Sigma-Aldrich (St. Louis, MO).

The Ub-EGFP reporter plasmid was constructed by inserting human ubiquitin and the linker sequence VGKLGQRQDPPVAT N-terminal to EGFP into pEGFP-N1 (Clontech, Mountain View, CA). The QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to change Gly76 into Val in ubiquitin and Met1 into Leu in EGFP.

Antibodies against GFP were from Roche Diagnostics (Mannheim, Germany), anti-GAPDH antibodies were from Chemicon International (Temecula, CA), and anti-phospho-GCN2 (phosphorylated at threonine 899) and anti-GCN2 antibodies were from Cell Signaling Technology (Beverly, MA).
Cell culture, transfection, and immunoblotting

Human HeLa and 293T cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Biochrom KG, Berlin, Germany), supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin G, 100 µg/ml streptomycin sulphate, 2 mM L-glutamine and non-essential amino acid cocktail (all from Gibco-Invitrogen). The medium used for experiments (incubation medium) was modified (Gibco-Invitrogen) to contain 20 µM L-Met and 80 µM L-Leu. Additionally, the incubation medium was devoid of FCS and was supplemented to contain all non-essential amino acids, 1 mM sodium pyruvate (Sigma) and 25 mM HEPES, pH 7.4 (Biomol). When indicated as being deficient, the incubation medium contained reduced amounts of L-Met (0.2 µM), L-Leu (0.8 µM), L-Phe (0.4 µM).

HeLa cells were transfected with 30 µg Ub-EGFP expression vector by electroporation in a 400 µl final volume (DMEM, 25% FCS) at 300 V and 950 µF with an electroporator GenePulser Xcell (Bio-Rad Laboratories, Hercules, CA). After transfection, cells were washed and subsequently incubated in normal serum-containing medium for 1.5-2 h.

Before performing kinetic experiments, cells were washed in the respective incubation medium (normal or deficient) and pre-warmed for 10-15 min. Aliquots were removed from the cultures at the times indicated upon addition of DMSO or inhibitors. For immunoblotting, aliquots containing 0.5 x10⁶ cells were mixed with pre-heated SDS sample buffer. Lysates were resolved on 10% (for GFP and GAPDH) or 7.5% (for phospho-GCN2) SDS-PAGE, and transferred onto Protran nitrocellulose membrane.
Membranes were blocked with 5% skim milk solution, probed with the indicated antibodies and developed using the chemiluminescence kit ECL plus (Amersham Biosciences, Buckinghamshire, UK).

Cell labeling

For radioactive labeling of newly synthesized proteins, cells were washed in the respective incubation medium (normal or deficient) and pre-warmed for 10-15 min. Aliquots were removed from the culture upon adding 50 μCi/ml $^{35}$S-Met or 100 μCi/ml $^{3}$H-Leu (both from Amersham Biosciences; 15 mCi/ml and 5 mCi/ml, respectively) together with DMSO or inhibitors (100 μM MG132, or 40 μM clasto-lactacystin-β-lactone, or 40μM epoxomicin). At the different time points aliquots of 0.5 x10$^6$ cells were added to an equal volume of 0.2% SDS/ 2 mg/ml BSA and immediately mixed with cold TCA to obtain a final TCA concentration of 10%. TCA precipitates were incubated for 30 min on ice, diluted 2-fold with water, and heated for 10 min at 90°C. Precipitates were captured on GF/C glass microfiber filters (Whatman, Middlesex, UK), washed first with 10% TCA, then with 100% ethanol, and finally dried. Counting was performed using scintillation fluid Rotiszint eco plus (Carl Roth, Karlsruhe, Germany) in a Packard-TRICARB1500 scintillation counter (PerkinElmer, Wellesley, MA) or LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). For gel analyses, cell suspension aliquots were lysed in an equal volume of 2X SDS sample buffer and run on a 10% SDS-PAGE gel. The gel was dried, exposed to a phosphorimager screen, scanned by a FLA-
2000 analyzer (Fuji Photo Film Co., Tokyo, Japan), and analyzed using AIDA software (Raytest, Straubenheim, Germany).

For double labeling, 50 \( \mu \text{Ci/ml} \) \( ^{35} \text{S-Met} \) (in Met-sufficient medium) or 5 \( \mu \text{Ci/ml} \) \( ^{35} \text{S-Met} \) (in Met-deficient medium) were used, the latter being reduced to keep radioactivity contributions from \( ^{35} \text{S-Met} \) and \(^{3} \text{H-Leu} \) similar. Scintillation counting was performed in two spectral windows to assign disintegrations to the respective isotopes.

**Amino acid analysis**

Cells were prepared as for nascent polypeptide labeling, except that the number of cells was doubled to match the detection sensitivity. Inhibitors were added at time 0, and 10 min later cells were harvested, washed in ice-cold PBS and lysed in 10\% TCA. TCA-soluble material was vacuum-dried and analyzed for amino acid content by the Max Planck Institute of Biochemistry chemical core facility using post-column derivatization with ninhydrin on a Biotronik LC 3000 amino acid analyzer.

**Methionyl-tRNA analysis**

Total RNA was isolated using the TRIzol reagent (Invitrogen). Aminoacyl-tRNA was hydrolyzed in 1\% \( \text{NH}_4\text{OH} \) at 37\(^{\circ}\)C for 30 min and released \(^{35} \text{S-Met} \) was separated on a thin-layer chromatography Silica gel 60 plate (Merck KGaA) with 12:3:5 butanol:acetic acid:water. The plate was dried and analyzed by phosphorimaging. \(^{35} \text{S-Met} \) spots were identified using \(^{35} \text{S-Met} \) standard solution. Radioactivity values were normalized to the respective amounts of isolated RNA.
Table S1. Proteasome activity replenishes the intracellular amino acid pool used for protein synthesis. Intracellular leucine levels were determined by amino acid analysis after incubation of HeLa cells in normal (80 µM Leu) or leucine-deficient (0.8 µM Leu) medium for 10 min in the presence of 100 µM MG132, 5 mM cyclohexamide (CHX), DMSO, or their combinations as indicated. Means ± SD of three independent experiments are shown. N.D., not detectable.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Leucine (nmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal medium</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>MG132</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>CHX / DMSO</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>CHX / MG132</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>Leu-deficient medium</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>N.D.</td>
</tr>
<tr>
<td>MG132</td>
<td>N.D.</td>
</tr>
<tr>
<td>CHX / DMSO</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>CHX / MG132</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Supporting Figures

**Figure S1.** Proteasome activity is required to sustain protein synthesis upon restriction of external amino acid supply. (A) Proteasome inhibition in HeLa cells with clasto-lactacystin-β-lactone (CL-β-lactone) causes reduced translation efficiency in leucine-deficient medium. Translation was followed by measuring the incorporation of $^{35}$S-Met into TCA-insoluble material. 50 μCi/ml $^{35}$S-Met and 40 μM of the inhibitor (solid symbols) or $^{35}$S-Met and DMSO (open symbols) were added at time 0. (B) Same as (A), except that 40 μM epoxomicin was used instead of CL-β-lactone. (C) Poteasome inhibition in 293T cells causes reduced translation efficiency in leucine-deficient medium. Translation was followed measuring the incorporation of $^{35}$C-Met into TCA-insoluble material. 50 μCi/ml $^{35}$S-Met and 100 μM MG132 (solid symbols) or $^{35}$S-Met and DMSO (open symbols) were added at time 0. Representative results of at least three independent experiments are shown.
Figure S2. Cytosolic degradation is required to sustain protein synthesis during acute decrease in external amino acid supply. (A) HeLa cells were incubated in Leu-deficient medium and newly synthesized proteins were labeled with 50 µCi/ml $^{35}$S-Met. Translation was followed by measuring the incorporation of $^{35}$S-Met into TCA-insoluble material. $^{35}$S-Met and 200 nM bafilomycin A1 (gray symbols), 100 µM chloroquine (black symbols) or DMSO (open symbols) were added at time 0. (B) Cells were incubated in normal medium (circles) or Leu-deficient medium (squares), and newly synthesized proteins were labeled with 50 µCi/ml $^{35}$S-Met. 100 µM bestatin methyl ester (bestatin ME, filled symbols) or DMSO (open symbols) were added together with $^{35}$S-Met at time 0. Right panel: Protein synthesis in cells 15 min after addition of inhibitor is expressed in % of controls lacking inhibitor. Means ± SD of three independent experiments.
Figure S3. Proteasome inhibition results in increased $^{35}$S-Met incorporation into newly synthesized proteins in methionine-deficient medium independently of the amount of $^{35}$S-Met added. During labeling in normal medium, the ratio of labeled to unlabeled amino acid (i.e. the specific radioactivity) is significantly lower than in Met-deficient medium. To exclude the possibility that this difference may influence the outcome of the experiment, cells were labeled in Met-deficient medium but the specific radioactivity was maintained constant at a ratio of 1 molecule of labeled Met to 400 molecules of unlabeled Met. Translation was followed measuring the incorporation of $^{35}$S-Met into TCA-insoluble material. Closed symbols, MG132; open symbols, DMSO. Inhibitor and radioactive tracer were added at time 0. One representative experiment of at least three independent experiments is shown.
Figure S4. Proteasome activity modulates the specific radioactivity of the intracellular amino acid pool used for protein synthesis. (A) Double labeling of newly synthesized proteins. Leu in medium was kept at normal concentration (80 µM), whereas the concentration of Met was varied (normal, 20 µM; - Met, 0.2 µM). Labeling was performed simultaneously with both $^3$H-Leu and $^{35}$S-Met as above. Specific activity (SA) refers to $^{35}$S-Met incorporation per unit of incorporated $^3$H-Leu during 10 min labeling in the presence of 100 µM MG132. SA was set to 1 for control cells receiving DMSO. Means ± SD of 3 experiments are shown. (B) Specific radioactivity of the methionyl-tRNA pool. The concentration of Met in the medium was varied as in (A). HeLa cells were pre-incubated for 15 min with 40 µM epoxomicin before adding 100 µCi/ml $^{35}$S-Met. Loading of the radioactive methionine onto tRNA was allowed to proceed for 90 sec in the presence of 5 mM CHX. Aminoacylated tRNA was determined as described in Materials and Methods. Specific activity was set to 1 for control cells receiving DMSO.
Means \( \pm \) SD of 3 independent experiments are shown. Identical results were obtained when MG132 was used instead of epoxomicin (data not shown).