Biochemical and Biophysical Research Communications 436 (2013) 41–46

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of ubiquitin-modified lysine residues and novel phosphorylation sites on eukaryotic initiation factor 2B epsilon

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ARTICLE INFO

Article history:
Received 29 April 2013
Available online 21 May 2013

Keywords:
mRNA translation
Protein synthesis
Proteasomal degradation
Posttranslational modification

ABSTRACT

Eukaryotic initiation factor 2Bε (eIF2Bε) plays a critical role in the initiation of mRNA translation and its expression and guanine nucleotide exchange activity are major determinants of the rate of protein synthesis. In this work we provide evidence that the catalytic epsilon subunit of eIF2B is subject to ubiquitination and proteasome-mediated degradation. Lysates of C2C12 myoblasts treated with proteasome inhibitor were subjected to sequential immunoprecipitations for eIF2Bε followed by ubiquitin. Tandem mass spectrometry (LC–MS/MS) analysis of immunoprecipitated proteins resulted in the identification of five peptides containing ubiquitin (diglycine) modifications on eIF2Bε. The specific lysine residues containing the ubiquitin modifications were localized as Lys-56, Lys-98, Lys-136, Lys-212 and Lys-500 (corresponding to the rat protein sequence). In addition three novel phosphorylation sites were identified including Ser-22, Ser-125, and Thr-317. Moreover, peptides corresponding to the amino acid sequence of the E3 ligase NEDD4 were also detected in the LC–MS/MS analysis, and an interaction between endogenous eIF2Bε with NEDD4 was confirmed by co-immunoprecipitation.

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1. Introduction

We recently reported that the catalytic epsilon subunit of the heteropentameric guanine nucleotide exchange factor eIF2B is subject to proteasome-sensitive degradation [1]. To examine the potential ubiquitin modification of eIF2Bε, we analyzed the primary protein sequence with bioinformatics algorithms. Using the UbiPred bioinformatics algorithm [2], 12 of the 27 lysines contained in the rat eIF2Bε protein were identified over the default threshold score of 0.50, with several being close to the highest prediction score of 1.0. In particular, one region contains a cluster of four lysines (Lys-470, Lys-472, Lys-474, Lys-476) with very strong prediction scores and the predictions were conserved in the mouse, rat and human proteins. Using the UbPred prediction algorithm [3], four lysines were the same as those predicted by UbiPred, with three additional potential sites at Lys-493, Lys-500, and Lys-688. Intriguingly, the region immediately N-terminal to the cluster of predicted ubiquitin sites contains a high scoring PEST motif, another potential signal for protein degradation [4]. Based on our previous report and the *in silico* predictions that several lysines contained within the eIF2Bε protein are putative sites of ubiquitin modification we performed the experiments described herein to verify and characterize such posttranslational modifications. The results demonstrate that eIF2Bε is indeed subject to ubiquitin modification and identify five lysine residues within the rat protein that are modified by ubiquitin as determined by tandem mass spectrometry. The results also identify three novel phosphorylation sites and implicate NEDD4 as the E3 ligase involved in the proteosome-mediated degradation of eIF2Bε.

2. Materials and methods

2.1. Cell culture and reagents

C2C12 myoblasts (ATCC) were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen) containing 25 mM glucose supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals), and 1% penicillin–streptomycin (Invitrogen), at 37 °C and 5% CO_2. MG-132 (CalBiochem/EMD Biosciences) was prepared as a 10 mM stock solution in DMSO and used at the concentration indicated in the figure legends.

2.2. Plasmids and transfections

The plasmid pFLAG-eIF2Bε in the pcDNA3.1 expression vector (Invitrogen) was generated from the previously cloned rat eIF2Bε cDNA [5] with an N-terminal FLAG-epitope [6]. The plasmid

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.05.053

pRK5-HA-Ubiquitin-WT, encoding wild type human ubiquitin C with an N-terminal HA epitope tag [7] was obtained from AddGene, where it was originally deposited by T.M. Dawson (The Johns Hopkins University School of Medicine). Transient transfection of C2C12 myoblasts was accomplished with the Effectene Transfection Reagent (Qiagen) utilizing a modified protocol [8]. Briefly, myoblasts were trypsinized on the day of transfection and treated as a suspension of cells with 1:8 DNA:Enhancer and 1:15 DNA:Effectene ratios (mass:volume), respectively.

2.3. SDS–PAGE and Western blot analysis

Cell lysates or immunoprecipitated proteins were resolved by SDS–PAGE and subjected to Western blot analysis as described previously [9]. Primary antibodies used included: anti-eIF2Bε (generated in house); anti-α-tubulin (Santa Cruz Biotechnology, #sc-32293); anti-DDK (Origene, #4C5); and anti-HA (Santa Cruz rabbit Biotechnology, #sc-805). After overnight incubation with primary antibody, membranes were probed with secondary antibodies (Bethyl Laboratories) in TBST with 5% non-fat dry milk for 1 h at room temperature. Blots were developed with ECL (Pierce/Thermo Scientific) or ECL Plus (GE Healthcare) detection reagents. Images were acquired with a GeneGnome HR imaging system and GeneSnap software (SynGene). In some cases, PVDF membranes were stripped and then re-probed with a different primary antibody.

2.4. Preparation of cell extracts

Unless otherwise noted, cells were lysed with RIPA buffer (Sigma–Aldrich) supplemented with (final concentrations): 1 mM dithiothreitol, 1 mM benzamidine, 0.5 mM sodium vanadate, and 10 μl/ml Sigma Protease Inhibitor Cocktail (Sigma–Aldrich). N-ethylmaleimide (NEM; Sigma–Aldrich) was added to a final concentration of 10 mM immediately before use. Cells were harvested using trypsin, collected by centrifugation at 233*g* for 5 min, washed with PBS, recentrifuged, and finally resuspended in lysis buffer. The cell suspension was rocked for 30 min at 4 °C followed by centrifugation at 8200*g* for 10 min at 4 °C. The cleared lysate was either combined with 2X SDS–PAGE sample buffer or subjected to immunoprecipitation as described below.

2.5. Sequential FLAG(eIF2Bε) and HA(ubiquitin) immunoprecipitation

Immunoprecipitation of FLAG-eIF2Bε covalently modified with HA-Ubiquitin was performed using the FLAG HA Tandem Affinity Purification kit (Sigma–Aldrich). Twenty 10-cm culture dishes were each seeded with 1.5×10^6 C2C12 myoblasts and simultaneously transfected with pFLAG-eIF2Bε (6.0 μg) and pRK5-HA-Ubiquitin-WT (2.0 μg) and 24 h later the cells were incubated in serum-free DMEM for 16 h. Cells were then treated with MG-132 (10 μM) for 8 h in serum-free DMEM. Cells were harvested in RIPA buffer supplemented with 10 mM NEM, 10 μM MG-132 and the inhibitors described above. The lysate was sequentially immunoprecipitated using the EZView anti-FLAG M2 affinity resin and the anti-HA agarose affinity resin using the manufacturer's protocol. Proteins were eluted from the HA resin with buffer consisting of 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue. Finally, β-mercaptoethanol (5% v/v) was added and the sample boiled at 100 °C for 5 min. A small portion of the eluate was used for immunoblot analysis, while the remainder was subjected to electrophoresis on a Criterion SDS–PAGE gel. Proteins were visualized with Bio-Safe Coomassie gel stain (Bio-Rad) and excised bands were sent for analysis at the Taplin Biological Mass Spectrometry Facility at Harvard University (Harvard Medical School, Boston, MA).

2.6. eIF2B – NEDD4 co-immunoprecipitation

Cell extracts were prepared as described above, and eIF2Bε was immunoprecipitated from an aliquot of the extract using an anti-eIF2Bε monoclonal antibody [10]. NEDD4 was immunoprecipitated from a separate aliquot of the same cell extract using an anti-NEDD4 antibody (Cell Signaling, #5344). Antibody-antigen complexes were collected using magnetic beads (Qiagen), and the beads were washed thrice with ice-cold buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% β-mercaptoethanol, pH 7.4), resuspended in 1× SDS sample buffer, and then boiled for 5 min. Supernatants were subjected to Western blot analysis using either anti-eIF2Bε or anti-NEDD4 antibody (Cell Signaling, #2740).

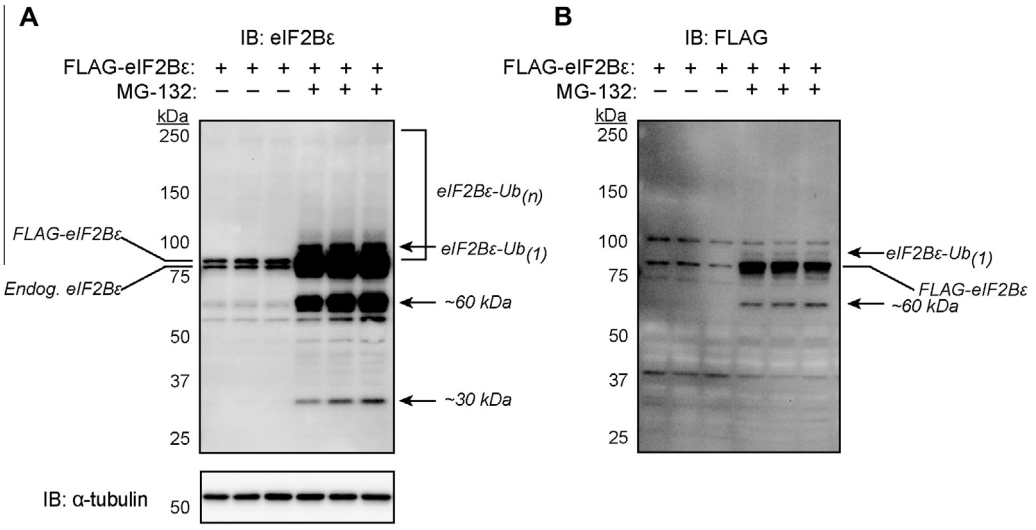

Fig. 1. Accumulation of FLAG-eIF2Bε with the proteasome inhibitor MG-132 and appearance of high molecular weight bands. (A and B) C2C12 myoblasts were transfected and treated with MG-132 as described under "Materials and methods". Equal volumes of the lysates were subjected to immunoblot (IB) analysis with antibodies against eIF2Bε, FLAG epitope, or α-tubulin. The doublet in panel A represents the endogenous (Endog) eIF2Bε and the expressed FLAG-eIF2Bε. Arrows indicate potentially ubiquitinated proteins as well as potential degradation products that accumulate with MG-132 treatment. The experiment was performed with three replicates for each time point and condition; representative blots are shown.

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