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The ECS(SPSB) E3 ubiquitin ligase is the master regulator of the lifetime of inducible nitric-oxide synthase

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ABSTRACT

The ubiquitin–proteasome pathway is an important regulatory system for the lifetime of inducible nitric-oxide synthase (iNOS), a high-output isoform compared to neuronal NOS (nNOS) and endothelial NOS (eNOS), to prevent overproduction of NO that could trigger detrimental effects such as cytotoxicity. Two E3 ubiquitin ligases, Elongin B/C–Cullin-5–SPRY domain- and SOCS box-containing protein [ECS(SPSB)] and the C-terminus of Hsp70–interacting protein (CHIP), recently have been reported to target iNOS for proteasomal degradation. However, the significance of each E3 ubiquitin ligase for the proteasomal degradation of iNOS remains to be determined. Here, we show that ECS(SPSB) specifically interacted with iNOS, but not nNOS and eNOS, and induced the subcellular redistribution of iNOS from dense regions to diffused expression as well as the ubiquitination and proteasomal degradation of iNOS, whereas CHIP neither interacted with iNOS nor had any effects on the subcellular localization, ubiquitination, and proteasomal degradation of iNOS. These results differ from previous reports. Furthermore, the lifetime of the iNOS(N27A) mutant, a form of iNOS that does not bind to ECS(SPSB), was substantially extended in macrophages. These results demonstrate that ECS(SPSB), but not CHIP, is the master regulator of the iNOS lifetime.

1. Introduction

Nitric oxide (NO) is an important multifunctional biomolecule that is endogenously synthesized from L-arginine by NO synthases (NOSs) [1]. Inducible NOS (iNOS) is known to produce a relatively large amount of NO because of its Ca^{2+}-independent activity [1]. Excessive NO production via iNOS induces apoptotic cell death of activated macrophages [2] and is linked to numerous human pathologies, including asthma, arthritis, and endotoxin shock [3,4]. Thus, the lifetime of iNOS needs to be tightly regulated. iNOS is known to be degraded by the ubiquitin–proteasome pathway [5,6]. The two E3 ubiquitin ligases, Elongin B/C–Cullin-5–SPRY domain- and SOCS box-containing protein [ECS(SPSB)] and the C-terminus of Hsp70–interacting protein (CHIP), have been reported to target iNOS for proteasomal degradation [7–10]. However, the significance of each E3 ubiquitin ligase for the ubiquitination and proteasomal degradation of iNOS has not been clarified.

Because CHIP has been shown to promote ubiquitination and proteasomal degradation of non-native or misfolded proteins rather than native or properly folded proteins [11–17], CHIP is involved in the regulation of a wide range of proteins. In addition, CHIP has been shown to regulate neuronal NOS (nNOS) and endothelial NOS (eNOS) as well as iNOS [18,19]. In contrast, the specificity of ECS(SPSB) is strict, because SPSB proteins, the substrate recognition subunits of ECS(SPSB), recognize unique core sequences, D/E-I/L-N-N-N [20]. Only 11 mouse proteins and 16 human proteins contain this sequence [10], and currently iNOS is the only substrate identified for ECS(SPSB). Whether or not ECS(SPSB) regulates nNOS and eNOS is unknown.

In the present study, we investigated both the specificity of ECS(SPSB) for all NOS isoforms and the relative significance of ECS(SPSB) and CHIP for ubiquitination and proteasomal degradation of iNOS. Our findings suggest that ECS(SPSB), but not CHIP, is an iNOS-specific E3 ubiquitin ligase and is the master regulator of the iNOS lifetime.

Abbreviations: ECS, Elongin B/C–Cullin-5–SOCS box protein; SPSB, SPRY domain- and SOCS box-containing protein; ECS(SPSB), ECS containing SPSB as a SOCS box protein; NOS, nitric-oxide synthase; iNOS, inducible NOS; CHX, cycloheximide; YFP, yellow fluorescent protein.

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2. Materials and methods

2.1. Reagents

Anti-Myc antibody was obtained from Cell Signaling Technology. Anti-FLAG (M2) antibody and anti-CHIP antibody were from Sigma. Anti-iNOS antibody was from Millipore. Anti-GAPDH antibody was from Santa Cruz Biotechnology. Anti-α-tubulin antibody was from Invitrogen. Anti-6× His-tag antibody was from MBL. Anti-GFP antibody (clone JL-8) was from Clontech. Cycloheximide (CHX) was from Calbiochem.

2.2. Cell culture

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HEK293T cells stably expressing myc-tagged ubiquitin (293T-^{myc}Ub cells [9]) were grown in DMEM containing 10% FBS and 1 μg/ml puromycin. RAW264.7 mouse macrophage cell line was grown in RPMI containing 10% FBS and 1 mM pyruvate.

2.3. cDNAs and plasmids

The cDNAs encoding full-length human iNOS (hiNOS), hnNOS and heNOS were subcloned into the pCMV-Tag5A vector (Stratagene). The plasmids for expressing hiNOS mutants (N26A, N27A, and N25–27A) were constructed by using the QuikChange™ Site-Direct Mutagenesis Kit (Stratagene) and pSG5-hiNOS vector as a template. The cDNAs encoding residues 1–263 (FL), 1–85 (N), 86–219 (SPRY), 220–263 (SOCS), 86–263 (ΔN), 1–85 fused to 220–263 (ΔSPRY), and 1–221 (ΔSOCS) of human SPSB2 (hSPSB2) were subcloned into the pGEX-6P-2 vector (GE Healthcare). The cDNAs encoding hiNOS and hiNOS(N27A) mutant were subcloned into the pMXrmv5-$(G_4S)_3$-YFP retroviral vector [21].

2.4. Co-immunoprecipitation

HEK293T cells in a 6-well plate were transfected with the indicated plasmids for 24 h. The cells were lysed in 500 μl of buffer A (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and a protease inhibitor mixture (Roche), pH 7.5). The lysates were centrifuged at 20,000*g* for 10 min at 4 °C. The supernatants were pre-cleared with 40 μl of protein G-Sepharose 4FF beads (GE Healthcare) for 30 min. The pre-cleared lysates were incubated with the indicated antibodies for 16 h at 4 °C, and successively with 40 μl of protein G-Sepharose 4FF beads for 4 h at 4 °C. The beads were washed five times with 1 ml of buffer A. Immunoprecipitated proteins were eluted by boiling with 40 μl of 2× SDS–PAGE sample buffer for 5 min, and subjected to immunoblotting.

2.5. GST-pull down assay

GST fusion proteins were expressed in BL21-CodonPlus(DE3)-RILP bacteria (Stratagene) and were purified by using the Glutathione Sepharose 4B (GE Healthcare) as described previously [22].

HEK293T cells were transfected with pSG5-hiNOS. After 24 h, lysates were prepared, and the supernatants (200 μg proteins) were incubated with 5 μg of GST fusion proteins for 3 h at 4 °C. The GST fusion protein-bound beads were washed five times with buffer A, boiled with 75 μl of 2× SDS–PAGE sample buffer, and 12 μl of each sample was subjected to immunoblotting using anti-iNOS antibody.

2.6. Quantitation of nitrite in culture medium

The production of nitrite was measured using Griess reagent as described previously [23].

2.7. Detection of ubiquitinated iNOS

293T-^{myc}Ub cells in 6-well plates were washed with PBS and lysed with 1 ml buffer B (PBS containing 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, 0.5 mM EDTA, 5 mM *N*-ethylmaleimide, 1 mM NaF, and a protease inhibitor cocktail). The lysates were centrifuged at 20,000*g* for 20 min at 4 °C, and the supernatants were then pre-cleared with 50 μl protein G-Sepharose 4FF beads for 30 min, and centrifuged at 20,000*g* for 10 min at 4 °C. The pre-cleared lysates were incubated with 3 μg of anti-iNOS antibody for 90 min at 4 °C, and successively with 50 μl protein G-Sepharose 4FF beads for 90 min at 4 °C. The beads were washed five times with 1 ml buffer B. Immunoprecipitated proteins were eluted by boiling with 40 μl 2× SDS–PAGE sample buffer for 1 min, and subjected to immunoblotting.

2.8. Microscopy

HEK293T cells transfected with the indicated plasmids were placed into a glass bottom dish (IWAKI) coated with poly-L-lysine (Sigma). The next day, images were acquired using an Olympus IX-71 fluorescent microscope.

2.9. Expression of YFP fusion proteins in RAW264.7 macrophages

Introduction of genes into RAW264.7 macrophages was carried out by retroviral gene transfer as described previously [23].

3. Results

3.1. Neither nNOS nor eNOS are regulated by ECS(SPSB)

We have recently reported that the ECS E3 ubiquitin ligase containing SPSB1, SPSB2, or SPSB4 as a SOCS box protein (ECS(SPSB)) targets iNOS for proteasomal degradation [9]. The SPSB recognition sequence (DINNN) is present in the N-terminal region of iNOS (amino acids 23–27) [10,20]. The N-terminal region located before the oxygenase domain of three NOS isoforms contains a domain or motif that is unique to each NOS isoform and thus endows each NOS isoform with specific biochemical and physiological features [24,25]. To examine whether ECS(SPSB) specifically regulates iNOS, we compared the amino acid sequence of N-terminal regions of three NOS isoforms and found that the DINNN motif is conserved in iNOS proteins from various animals (data not shown), but is not present in either nNOS or eNOS (Fig. 1A). Consistent with this finding, iNOS interacted with SPSB2 (Fig. 1B) and was rapidly degraded in the presence of SPSB1 (Fig. 1C), whereas neither nNOS nor eNOS interacted with SPSB2 and were degraded despite SPSB1 expression. These results demonstrate that ECS(SPSB) specifically regulates iNOS.

The authors have previously reported that asparagine 27 of iNOS is a key residue for interactions with SPSB1, SPSB2, and SPSB4 [9,10]. However, it remains unclear which part of SPSB is involved in the interaction with iNOS. To map the iNOS binding site on SPSB, we generated selective deletions of the SPSB2 sequence (Fig. 1D), and analyzed the interaction with iNOS by GST pull-down assays. We found that the entire molecule, except the SOCS box, is required for the interaction with iNOS, although the SPRY domain alone faintly bound to iNOS (Fig. 1E).