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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).

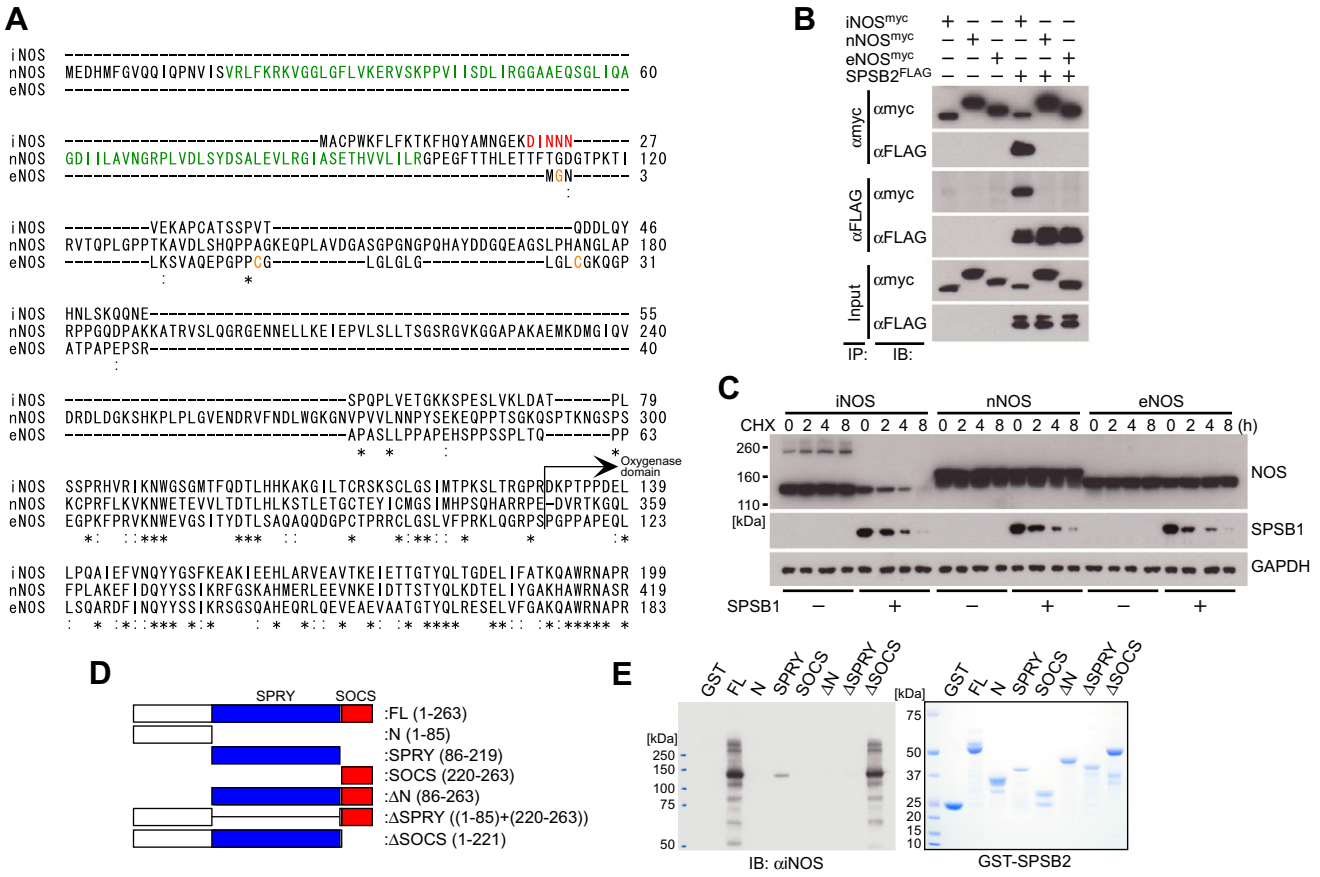

Fig. 1. iNOS, but not nNOS and eNOS, interacts with ECS(SPSB) and is rapidly degraded by ECS(SPSB). (A) Alignment of amino acid residues at the N-terminal region of NOSs. Amino acid residues at the N-terminal region of human iNOS (GenBank: AAI30284), human nNOS (GenBank: NP_000611), and human eNOS (GenBank: ABY87544) were aligned by introducing gaps (–) to obtain maximum homology. The SPSB recognition motif in iNOS is shown in red, the PDZ domain in nNOS is shown in green, and both the myristoylation site (Gly-2) and palmitoylation sites (Cys-15 and -26) in eNOS are shown in orange. A colon indicates amino acid similarity and a star indicates amino acid identity. (B) Interactions between SPSB2 and three NOS isoforms. HEK293T cells in 6-well plate were transfected with expression plasmids for FLAG-tagged SPSB2 (1 μg) and myc-tagged NOS isoforms (0.5 μg) for 24 h. The empty vector was also transfected to ensure that a total of 4 μg of DNA was used per transfection. The cell lysates were prepared and subjected to immunoprecipitation and immunoblotting. (C) HEK293T cells in 6-well plate were transfected with expression plasmids for FLAG-tagged SPSB1 (1 μg) and myc-tagged NOS isoforms (0.75 μg) for 12 h. The empty vector was also transfected to ensure that a total of 4 μg of DNA was used per transfection. Then the cells were placed into 4 wells of a 24-well plate. After 12 h, the cells were treated with 100 μM CHX for the indicated periods. The cell lysates were prepared and subjected to immunoblotting. (D) A schematic representation of SPSB2 mutants. (E) An immunoblot of an iNOS pull-down with various GST-tagged SPSB2 fragments bound to Glutathione Sepharose 4B (left panel). The amounts of each GST-tagged SPSB2 fragment were estimated from Coomassie blue staining (right panel).

3.2. ECS(SPSB), but not CHIP, regulates the subcellular redistribution and proteasomal degradation of iNOS

CHIP is the first E3 ubiquitin ligase that has been reported to promote the ubiquitination and proteasomal degradation of iNOS [7,8]. To determine which E3 ubiquitin ligase, ECS(SPSB) or CHIP, is essential for iNOS regulation, we examined the down-regulation of iNOS levels when SPSB1 or CHIP was co-expressed. We found that both the iNOS level and NO production via iNOS were substantially decreased when SPSB1 was co-expressed, whereas the co-expression of CHIP affected neither the iNOS level nor NO production via iNOS (Fig. 2 A and B), which differs from previous observations [7,8]. We next examined the stability of iNOS proteins in cycloheximide (CHX) chase assays [26]. We found that SPSB1 triggered iNOS degradation, and in particular, that the much lower expression of SPSB1 induced more rapid degradation of iNOS (Fig. 2C), consistent with our previous report [9]. In contrast, CHIP did not induce the degradation of iNOS, though CHIP down-regulated the basal levels of all proteins tested, especially GAPDH, in a dose-dependent manner.

Next, we examined the interactions of iNOS with SPSB1 and CHIP. FLAG-tagged SPSB1 and 6× His-tagged CHIP were expressed together with iNOS in HEK293T cells, and then those proteins were immunoprecipitated with antibodies for iNOS, FLAG-tag, and 6× His-tag. Co-immunoprecipitation with each protein was determined by immunoblotting. We found that SPSB1 was co-immunoprecipitated with iNOS in anti-iNOS immunoprecipitants (Fig. 2D, lane 5) and iNOS was also co-immunoprecipitated with SPSB1 in anti-FLAG immunoprecipitants (Fig. 2E), suggesting that iNOS interacts with SPSB1. In contrast, the interaction between iNOS and CHIP was not detected in either anti-iNOS or anti-6× His-tag immunoprecipitants (Fig. 2 D and F). Although a faint signal was seen under overexposure conditions on an anti-CHIP immunoblot for anti-iNOS immunoprecipitants (Fig. 2D, lane 6), it was uncertain whether this signal was derived from CHIP or not, because a similar signal was also observed in control IgG immunoprecipitants (Fig. 2D, lanes 1–3). Consistent with the fact that CHIP was originally identified as a protein interacting with Hsp70 [17], we found that endogenous Hsp70 was co-immunoprecipitated with 6× His-tagged CHIP in anti-6× His-tag immunoprecipitants (Fig. 2F), suggesting that 6× His-tagged CHIP functioned properly.

Given that we have recently reported that SPSB1, SPSB2, and SPSB4 can induce the subcellular redistribution of iNOS from dense regions to diffused expression [9], we next examined whether the

subcellular localization of iNOS is affected by CHIP. As shown in Fig. 2G, the subcellular localization of C-terminal yellow fluorescent protein (YFP)-tagged iNOS was unaffected by the co-expression of CHIP.

Finally, we investigated the levels of ubiquitinated iNOS when SPSB1 or CHIP was expressed. To evaluate the level of ubiquitinated iNOS, HEK293T cells stably expressing myc-tagged ubiquitin were transfected with cDNAs expressing iNOS, SPSB1, and CHIP for 24 h, followed by treatment with MG-132 for 4 h to accumulate ubiquitinated proteins. iNOS was immunoprecipitated with an anti-iNOS antibody and then the levels of ubiquitinated iNOS were analyzed by immunoblotting using an anti-myc antibody. We confirmed the expression of transfected iNOS, SPSB1, and CHIP by immunoblotting (Fig. 3A). We found that the level of ubiquitinated iNOS was enhanced more than 3-fold in cells transfected with SPSB1, whereas it was unchanged in cells transfected with CHIP (Fig. 3B and C). Taken together, our data demonstrate that CHIP is not involved in the regulation of iNOS.

3.3. The lifetime of the iNOS(N27A) mutant is much longer than that of wild-type iNOS in macrophages

Because the iNOS(N27A) mutant, a form of iNOS that does not bind to ECS(SPSB), is completely resistant to the protein degradation mediated by ECS(SPSB) [9], the amounts of ECS(SPSB) and CHIP critical for the proteasomal degradation of iNOS could be determined by the degree of degradation of the iNOS(N27A) mutant. Thus, we examined the degradation rates of wild-type iNOS and the iNOS(N27A) mutant in RAW264.7 macrophages, in which endogenous SPSB1, SPSB2, and CHIP are expressed [8,9]. YFP, iNOS–YFP, or the iNOS(N27A)–YFP were retrovirally expressed in RAW264.7 macrophages and the stabilities of those proteins were examined in a CHX chase assays. Approximately 80% of iNOS–YFP was degraded within 4 h after CHX treatment, whereas approximately 70% of the iNOS(N27A)-YEP was still present 8 h after CHX treatment (Fig. 4A), with a similar kinetic of degradation of YFP alone (Fig. 4B). These data suggest that the ECS(SPSB) E3 ubiquitin ligase is the master regulator of the iNOS lifetime in macrophages.

4. Discussion

We performed experiments to investigate whether ECS(SPSB) specifically regulates iNOS among all NOS isoforms, and to determine the degree to which ECS(SPSB) and CHIP are fundamental to iNOS regulation. Our results show that ECS(SPSB) binding is iNOS-specific, and ECS(SPSB) targets iNOS, but not nNOS and eNOS, for proteasomal degradation. In addition, the lifetime of the iNOS(N27A) mutant, a form of iNOS not bound to ECS(SPSB), is substantially extended in macrophages. In contrast, CHIP had

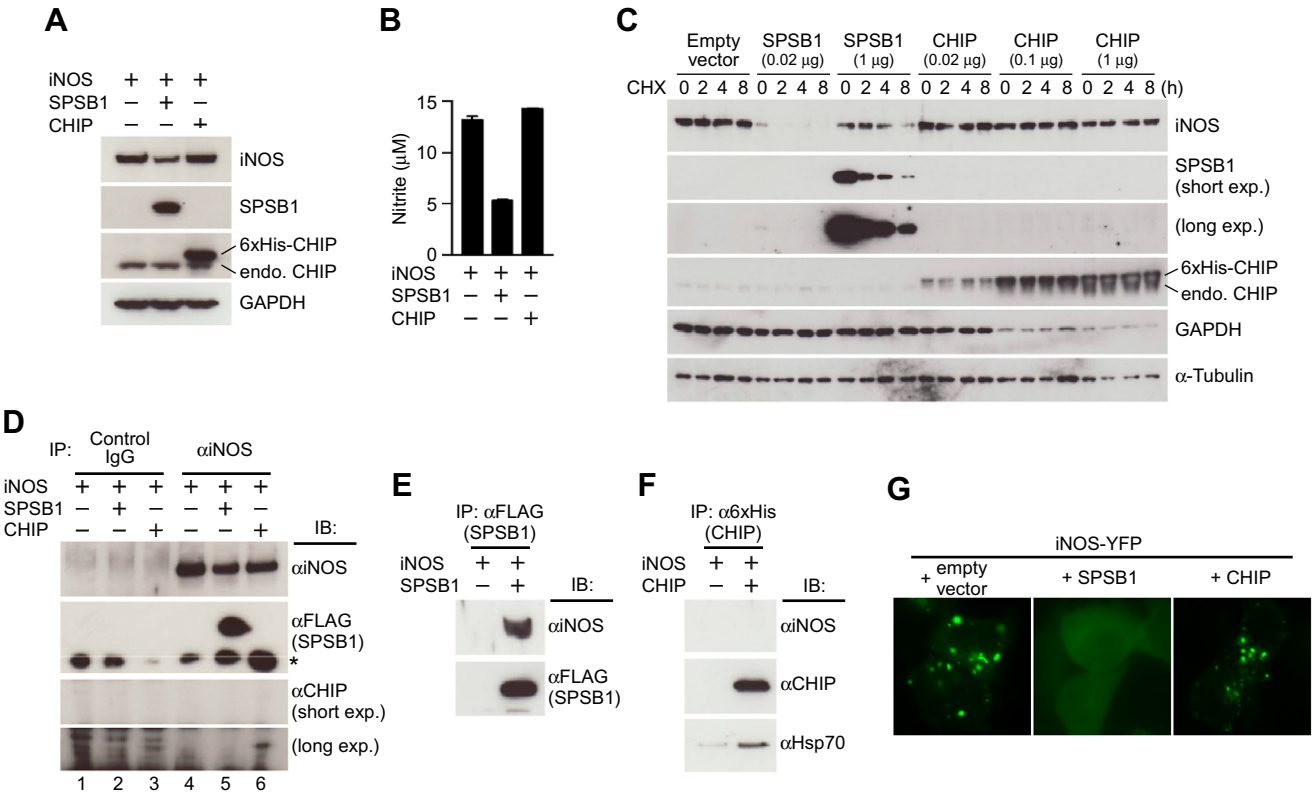

Fig. 2. ECS(SPSB), but not CHIP, regulates the subcellular redistribution and proteasomal degradation of iNOS (A) and (B), HEK293T cells in 6-well plates were transfected with expression plasmids for iNOS (2 μg) and FLAG-tagged SPSB1 (0.25 μg) or 6× His-tagged CHIP (0.25 μg) for 24 h. The empty vector was also transfected to ensure that a total of 4 μg of DNA was used per transfection. Then, the cell lysates were prepared, and subjected to immunoblotting using anti-iNOS, anti-FLAG, anti-CHIP, and anti-GAPDH antibodies. The concentration of nitrite in the cell culture medium was assessed by the Griess assay (B). Data are presented as mean ± standard deviation (SD), $n = 3$. (C) HEK293T cells in 6-well plates were transfected with expression plasmids for iNOS (2 μg) and FLAG-tagged SPSB1 (0.02 and 1 μg) or 6× His-tagged CHIP (0.02, 0.1, and 1 μg) for 12 h. The empty vector was also transfected to ensure that a total of 4 μg of DNA was used per transfection and the cells were placed into 4 wells of 24-well plate. After 12 h, the cells were treated with 100 μM CHX for the indicated periods. The cell lysates were prepared and subjected to immunoblotting. (D–F) The lysates from A (800 μg proteins) were subjected to immunoprecipitation and immunoblotting. A star indicates the light chain of the antibody. (G) In 12-well plates, HEK293T cells were transfected with expression plasmids for iNOS–YFP (1 μg) and FLAG-tagged SPSB1 (0.1 μg) or 6× His-tagged CHIP (0.1 μg) for 12 h. The empty vector was also transfected to ensure that a total of 1.6 μg of DNA was used per transfection. The cells were then placed into a 35 mm glass bottom dish. The next day, the subcellular localization of iNOS–YFP proteins was examined by fluorescence microscopy.

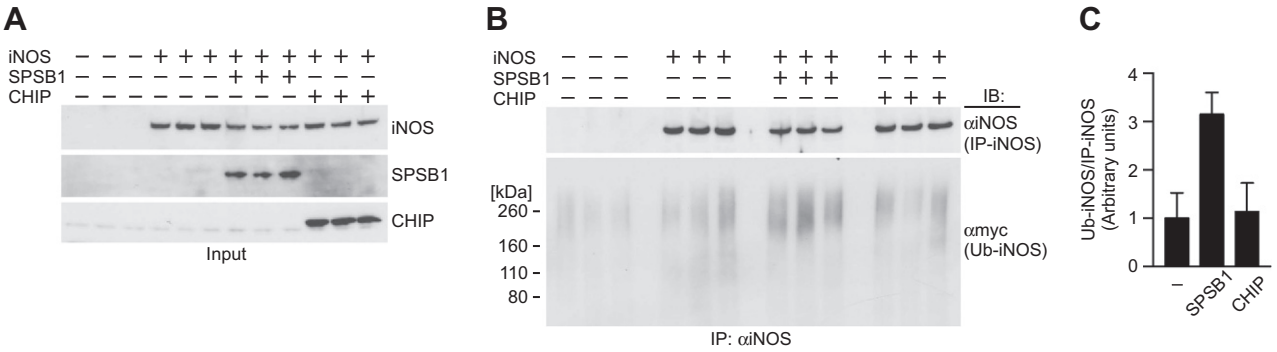

Fig. 3. ECS(SPSB), but not CHIP, induces the ubiquitination of iNOS. (A, B) In 6-well plates, 293T-^{myc}Ub cells were transfected with expression plasmids for iNOS (2 μg) and FLAG-tagged SPSB1 (0.1 μg) or 6× His-tagged CHIP (0.1 μg) for 24 h followed by treatment with 5 μM MG-132 for 4 h. A portion of each cell lysate was subjected to immunoblotting (A). The remaining cell lysate was subjected to immunoprecipitation using an anti-iNOS antibody, and the ubiquitinated iNOS was analyzed by immunoblotting using an anti-myc antibody (B). Each sample was prepared and loaded in triplicate. (C) The quantification of the levels of ubiquitinated iNOS shown in (B). Data represent mean ± SD, $n = 3$.

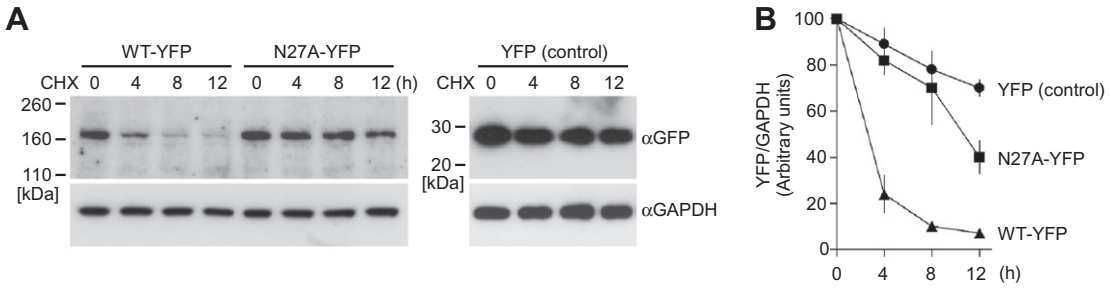

Fig. 4. The lifetime of the iNOS(N27A) mutant, a form of iNOS that does not bind to ECS(SPSB), is significantly extended in macrophages. (A) RAW264.7 macrophages were infected with retroviruses carrying YFP alone, iNOS–YFP, or the iNOS(N27A)-YFP. After 12 h, the cells were placed into 4 wells of 24-well plate. After 12 h, the cells were treated with 100 μM CHX for the indicated periods. The cell lysates were prepared and subjected to immunoblotting. (B) The quantification of the levels of YFP, iNOS–YFP, and the iNOS(N27A)-YFP shown in (A). Data represent mean ± SD, $n = 3$.

absolutely no effect on the regulation of iNOS. These results demonstrate that ECS(SPSB), but not CHIP, is an essential E3 ubiquitin ligase for the regulation of the iNOS lifetime.

Eissa et al. first reported the ubiquitin/proteasome-dependent degradation of iNOS [5,6,27]. Furthermore, Eissa et al. as well as Yin et al. independently reported that CHIP facilitates the ubiquitination and proteasomal degradation of iNOS [7,8]. In addition, we, along with Nicholson et al. recently reported that ECS(SPSB) targets iNOS for proteasomal degradation [9,10]. In contrast to the previous reports by Eissa et al. and Yin et al., in the present study we did not see any effects of CHIP on iNOS regulation in terms of the interaction with iNOS, the subcellular redistribution of iNOS, and ubiquitin/proteasome-dependent degradation of iNOS. Many reports have demonstrated that CHIP promotes ubiquitination and proteasomal degradation of non-native or misfolded proteins, rather than native or properly folded proteins [11–17]. The previous findings that CHIP mediated the proteasomal degradation of iNOS may have resulted from the expression of iNOS proteins as misfolded proteins in the respective experimental systems. In this study, iNOS was expressed in HEK293T cells by transfection with pSG5–hiNOS plasmid at physiological levels, because the iNOS levels expressed by this plasmid were quite similar to those expressed in RAW264.7 macrophages stimulated with 10 ng/ml LPS for 24 h (data not shown). In this case, most iNOS proteins are present in detergent soluble fractions (data not shown). It is possible that the previous studies used iNOS expression constructs that carry much stronger promoters, and therefore a portion of the iNOS proteins were misfolded and recognized as such by CHIP.

Currently, iNOS is the only substrate identified for ECS(SPSB). SPSB1, SPSB2, and SPSB4 recognize unique core sequences, D/E-I/L-N-N-N [20,28]. We performed a BLAST search to search for any proteins containing those sequences. A total of 17 human proteins were identified, only 4 of which contain those sequences across species, suggesting that ECS(SPSB) functions mainly to maintain appropriate iNOS levels through the ubiquitin/proteasome-dependent degradation of iNOS to prevent overproduction of NO during iNOS induction. Further studies using iNOS(N27A) knock-in mice may more clearly determine the significance of ECS(SPSB) E3 ubiquitin ligase in the regulation of the iNOS lifetime.

Conflict of interest

None.

Acknowledgments

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