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Regulation of Inducible Nitric-oxide Synthase by the SPRY Domain- and SOCS Box-containing Proteins*[S]

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Inducible nitric-oxide synthase (iNOS, NOS2) plays a prominent role in macrophage bactericidal and tumoricidal activities. A relatively large amount of NO produced via iNOS, however, also targets the macrophage itself for apoptotic cell death. To uncover the intrinsic mechanisms of iNOS regulation, we have characterized the SPRY domain- and SOCS box-containing protein 1 (SPSB1), SPSB2, and SPSB4 that interact with the N-terminal region of iNOS in a D-I-N-N-N sequence-dependent manner. Fluorescence microscopy revealed that these SPSB proteins can induce the subcellular redistribution of iNOS from dense regions to diffused expression in a SOCS box-dependent manner. In immunoprecipitation studies, both Elongin C and Cullin-5, components of the multi-subunit E3 ubiquitin ligase, were found to bind to iNOS via SPSB1, SPSB2, or SPSB4. Consistently, iNOS was polyubiquitinated and degraded in a proteasome-dependent manner when SPSB1, SPSB2, or SPSB4 was expressed. SPSB1 and SPSB4 had a greater effect on iNOS regulation than SPSB2. The iNOS N-terminal fragment (residues 1–124 of human iNOS) could disrupt iNOS-SPSB interactions and inhibit iNOS degradation. In lipopolysaccharide-treated macrophages, this fragment attenuated iNOS ubiquitination and substantially prolonged iNOS lifetime, resulting in a corresponding increase in NO production and enhanced NO-dependent cell death. These results not only demonstrate the mechanism of SPSB-mediated iNOS degradation and the relative contributions of different SPSB proteins to iNOS regulation, but also show that iNOS levels are sophisticatedly regulated by SPSB proteins in activated macrophages to prevent overproduction of NO that could trigger detrimental effects, such as cytotoxicity.

Ubiquitination and the subsequent proteasomal degradation of regulatory proteins represent a key mechanism for various cellular processes, including cell cycle control (1), apoptosis (2), cellular stress regulation (3), and antigen presentation (4). The ubiquitination of proteins is performed by a set of three enzymes: E1, E2, and E3 (5). E3 is a ubiquitin ligase that confers specificity to ubiquitination by recognizing target substrates and mediating the transfer of ubiquitin from E2 to the substrate. The E3 ubiquitin ligases are a large, diverse group of proteins, and many of them can act alone, but some RING-type E3 ubiquitin ligases, such as the ECS[3] (Elongin B/C-Cullin-5 (Cul5)-SOCS (suppressor of cytokine signaling)-box protein)-type E3 ubiquitin ligase, are found as components of much larger multi-protein complexes (6). The SOCS box is ~40 residues in length and contains a BC box and a Cul5 box, which binds Elongin C and Cul5, respectively (7). The SOCS box has been found in more than 50 proteins encompassing nine different families that are defined by the type of domain or motif they possess upstream of the SOCS box (6, 8). The SPRY domain- and SOCS box-containing proteins, SPSB1 to SPSB4 (also known as SSB-1 to SSB-4), are characterized by a central SPRY domain essential for protein interaction and a C-terminal SOCS box, suggesting that SPSB proteins may function as part of the ECS E3 ubiquitin ligase complex with the SPRY domain determining the substrate for ubiquitination (9). It has been shown that the *Drosophila* SPSB, called "GUSTAVUS," interacts with the DEAD-box RNA helicase VASA (10), and human SPSB1, SPSB2, and SPSB4 interact with human prostate apoptosis response protein-4 (hPar-4) (11). The identification of similar sequences in VASA (D-I-N-N-N) and hPar-4 (E-L-N-N-N) suggests that the SPRY domains of SPSB1, SPSB2, and SPSB4 likely recognize a common peptide epitope. In contrast to the extensive analysis of SPRY domain structures, the biochemical and physiological functions of the SPSB family of proteins remain largely unknown.

Nitric oxide (NO) is an important multifunctional biomolecule involved in various physiological and pathological processes (12, 13). NO is endogenously synthesized from L-argin-

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[3] The abbreviations used are: ECS, Elongin B/C–Cullin-5-SOCS box protein; SOCS, suppressor of cytokine signaling; SPSB, SPRY domain- and SOCS box-containing protein; NOS, nitric-oxide synthase; iNOS, inducible NOS; YFP, yellow fluorescent proteins; CFP, cyan fluorescent protein; 7-AAD, 7-aminoactinomycin D; CHX, cycloheximide; DOX, doxycycline.

ine by NO synthases (NOSs), a family of enzymes that currently includes three different isoenzymes in mammals (14). The inducible isoform of NOS (iNOS or NOS2) is known to produce a relatively large amount of NO because of its Ca^{2+}-independent activity (14), and thus has been linked to numerous human pathologies, including Alzheimer disease, asthma, cancer, cerebral infarction, inflammatory bowel disease, arthritis, and endotoxin shock (15, 16). Thus, NO production by iNOS needs to be tightly regulated. iNOS activity can be controlled through the regulation of its synthesis, catalytic activity, and degradation. Once iNOS is expressed, its degradation is the most critical option left for the regulation of iNOS activity. Eissa's group (17–19) has reported that iNOS is regulated by ubiquitination and the proteasomal degradation pathway. Additionally, Kuang *et al.* (20) recently reported that iNOS interacted with SPSB2, and the half-life of iNOS was not only shortened in SPSB2-transgenic macrophages but also extended in SPSB2-deficient macrophages. These studies suggest that SPSB2 regulates the ubiquitination and proteasomal degradation of iNOS. The detailed mechanism by which SPSB2 regulates iNOS, however, and whether other proteins of the SPSB family also play a role in regulating iNOS remain to be determined.

The functions that are unique to each NOS isoform are attributed to the N-terminal region because the N-terminal regions, located before the oxygenase domain of three NOS isoforms, share remarkably low sequence homology and contain a unique domain or motif that enables each NOS isoform to interact with a specific partner (21) or to achieve a distinctive subcellular distribution (22). In the present study, we characterized various biological processes of iNOS regulation by the SPSB family of proteins that interact specifically with the N-terminal region of iNOS. Here, we report on the molecular mechanism by which the SPSB family of proteins targets iNOS to proteasomal degradation, the relative contributions of different SPSB proteins to iNOS regulation, and the physiological significance of SPSB-mediated iNOS degradation in activated macrophages.

EXPERIMENTAL PROCEDURES

Reagents—Ultra pure LPS from Salmonella Minnesota R595 (Re) was obtained from Alexis Biochemicals. 1400W and cycloheximide were from Calbiochem. Doxycycline was from Clontech. Anti-iNOS antibody was from Millipore. Anti-FLAG(M2) antibody was from Sigma. Anti-HA antibody was from Covance. Antibodies to Cul5, COX-2, and GAPDH were from Santa Cruz Biotechnology. Anti-JAK2 antibody and anti-Myc antibody were from Cell Signaling Technology. Anti-T7 antibody was from Novagen. Anti-ubiquitin antibody (FK2) was from Nippon Bio-Test Laboratories.

cDNAs and Plasmids—cDNAs for human iNOS (hiNOS) and mouse iNOS (miNOS) were kindly provided by H. Esumi and C. Nathan, respectively. cDNAs for human SPSB1 (hSPSB1), hSPSB3, and hSPSB4 were kindly provided by G. Wu (23). cDNA encoding the residues 7–263 of hSPSB2 was isolated from the positive yeast clone in yeast two-hybrid screening, and then cDNA encoding the full-length hSPSB2 was amplified by PCR with the following primers: 5′-CGCGGATCCGCCGCCATGGGCCAGACAGCTCTGGCAGGGGGCAGCAGCAGCAC-3′ and 5′-CCGGAATTCCCTGGTAGAGCAGGTAGCGCTTC-3′. The cDNAs encoding residues 1–124 and 1–500 of hiNOS were subcloned into the pGBKT7 vector (Clontech). The plasmid for expressing hiNOS carrying N27A mutation (hiNOS(N27A)) was constructed by using the QuikChange™ Site-directed Mutagenesis kit (Stratagene) and pSG5-hiNOS vector as a template. The cDNAs encoding hSPSB1, hSPSB2, hSPSB3, hSPSB4, hSPSB1ΔSB (residues 1–231), hSPSB2ΔSB (residues 1–221), and hSPSB4ΔSB (residues 1–231) were subcloned into the pEFBOSEX-FLAG vector (24). The cDNAs encoding hSPSB4, hiNOS(1–124) and hiNOS(1–124)(N27A) were subcloned into the pEFBOSEX-HA vector (24). The cDNAs encoding N-terminal Myc-tagged ubiquitin and 3xT7-tagged Elongin C were subcloned into the pMXrmv5 retroviral vector (25). The cDNAs encoding hiNOS and hiNOS(N27A) were subcloned into the pMXrmv5-$(G_4S)_3$-YFP retroviral vector (25). The cDNAs encoding hSPSB1 and hSPSB1ΔSB were subcloned into the pMXrmv5-$(G_4S)_3$-CFP retroviral vector (25). The cDNA encoding the residues 1–118 of miNOS (miNOS(1–118)) and miNOS(1–118)(N27A) with the C-terminal FLAG tag were subcloned into the pRevTRE vector (Clontech).

Generation of Stable Cell Lines—HEK293T cells expressing N-terminal Myc-tagged ubiquitin (HEK293T-^{myc}Ub cells) and N-terminal 3xT7-tagged Elongin C (HEK293T-^{3xT7}EloC cells) were prepared by retroviral gene transfer as described previously (25). The positive cells were selected for growth in medium containing 2 μg/ml puromycin for a week, and individual clones were tested by immunoblotting using an anti-Myc or an anti-T7 antibody. The DOX-regulated miNOS(1–118)^{FLAG}-expressing RAW264.7 mouse macrophage cell line (RAW-TetOFF-miNOS(1–118)^{FLAG} and -miNOS(1–118)(N27A)^{FLAG} cells) was prepared using the Rev-Tet System (Clontech). Briefly, the pRevTet-OFF vector was introduced to RAW264.7 cells by retroviral gene transfer as described previously (26). The positive cells were selected for growth in medium containing 400 μg/ml G-418 for 2 weeks. Individual clones were infected with the pRevTRE-Luc vector, and the "on-off" regulation by DOX was tested using a luciferase assay. The cells obtained at this step are referred to as RAW-TetOFF cells. Then the pRevTRE-miNOS(1–118)^{FLAG} or pRevTRE-miNOS(1–118)(N27A)^{FLAG} vector was introduced to the RAW-TetOFF cells by retroviral gene transfer and the positive cells were selected for growth in medium containing 400 μg/ml hygromycin B and 400 μg/ml G-418 for 2 weeks. The "on-off" regulation by DOX in each clone was tested by immunoblotting using an anti-FLAG antibody.

Cell Culture—HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HEK293T-^{myc}Ub cells and HEK293T-^{3xT7}EloC cells were grown in DMEM containing 10% FBS and 1 μg/ml puromycin. RAW-TetOFF-miNOS(1–118)^{FLAG} and RAW-TetOFF-miNOS(1–118)(N27A)^{FLAG} cells were grown in RPMI containing 10% FBS, 1 mM pyruvate, 200 μg/ml G-418, 200 μg/ml hygromycin B, and 100 ng/ml DOX. When proteins of interest were induced by removing DOX from the culture medium, Tet System Approved FBS (Clontech) was used

instead of regular FBS, because regular FBS may contain tetracycline.

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). The human liver, spleen, heart, fetal brain, and adult brain cDNA libraries (Clontech) were screened with amino acids 1–124 of human iNOS as the bait, following the manufacturer's instructions.

Quantitation of Nitrite in Culture Medium—The production of nitrite was measured using Griess reagent as described previously (26).

Co-immunoprecipitation—Both HEK293T cells and HEK293T-^{3xT7}EloC cells in a 6-well plate were transfected with the indicated plasmids for 24 h. The cells were lysed in 1 ml 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 4 h to overnight 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.

Detection of Ubiquitinated iNOS—Either HEK293T-^{myc}Ub cells in 6-well plates, or RAW-TetOFF-miNOS(1–118)^{FLAG} and RAW-TetOFF-miNOS(1–118)(N27A)^{FLAG} cells in 6-cm dishes were washed with PBS and lysed with 1 ml of buffer B (PBS containing 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, 0.5 mM EDTA, 5 mM *N*-ethylmaleimide (NEM), 1 mM NaF, and a protease inhibitor mixture). 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 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 of buffer B. Immunoprecipitated proteins were eluted by boiling with 40 μl of 2× SDS-PAGE sample buffer for 1 min, and subjected to immunoblotting.

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 a KEYENCE BZ-9000 or an Olympus IX-71 fluorescent microscope.

Cell Viability Assay—RAW-TetOFF-miNOS(1–118)^{FLAG} and RAW-TetOFF-miNOS(1–118) (N27A)^{FLAG} cells were washed with PBS, and then incubated with PBS containing 5 mM EDTA for 7 min. The cells were collected and stained with 100 μl of 0.25 μg/ml 7-aminoactinomycin D (7-AAD) in SB (PBS containing 1% FBS and 0.09% sodium azide) for 10 min in the dark. Then, 500 μl of SB was added to the cells, and the stained cells were analyzed by flow cytometry using FACSCalibur (BD) and FlowJo software (Tree Star).

Statistical Analysis—All data are presented as means and S.D. Probability values were based on a paired *t* test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

RESULTS

SPSB1 and SPSB4 Interact with iNOS in a D-I-N-N-N Sequence-dependent Manner and Induce Its Subcellular Redistribution in a SOCS Box-dependent Manner—To uncover the molecular mechanisms of iNOS regulation, we sought to identify proteins that interacted with the N-terminal region located before the oxygenase domain of human iNOS using yeast two-hybrid screens. Using residues 1–124 of human iNOS as the bait, we screened 3 × 10^7 independent clones from human liver, spleen, heart, fetal brain, and adult brain cDNA libraries and obtained three X-α-Gal positive clones. Sequencing analysis and Blast searches of the GenBank™ databases indicated that one of these clones encoded almost full-length SPSB2 (residues 7–263; supplemental Fig. S1). Kuang *et al.* (10, 20) recently bioinformatically identified iNOS as an SPSB2-interacting protein by searching for proteins that contained putative *Drosophila* SPSB recognition sequences ((D/E)-(I/L)-N-N-N). They showed that the D-I-N-N-N motif was present in residues 23–27 of iNOS proteins from different species and that asparagine 27 is a key residue for interacting with iNOS. They also demonstrated that SPSB1 and SPSB4, but not SPSB3, interacted with iNOS. However, whether SPSB1 and SPSB4 also recognize the D-I-N-N-N sequence remains unclear. Thus, we examined the interaction of the iNOS protein carrying the N27A mutation (iNOS(N27A)) with SPSB1 and SPSB4. In co-immunoprecipitation experiments, SPSB1 and SPSB4 interacted with wild-type iNOS, while neither interacted with the iNOS(N27A) mutant (Fig. 1*A*), suggesting that the iNOS interactions with SPSB1 and SPSB4 are regulated in the same manner as the interaction with SPSB2.

We used microscopic imaging to investigate the effects of SPSB proteins on the subcellular distribution of iNOS in living cells. We expressed iNOS fused at its C terminus to yellow fluorescent protein (YFP) and SPSB proteins fused at their C terminus to cyan fluorescent protein (CFP) in HEK293T cells, and imaged the cells using fluorescence microscopy. Both iNOS and the iNOS(N27A) mutant were localized as discrete foci scattered throughout the cytosol, while SPSB1 and the SOCS box-deleted form of SPSB1 (SPSB1ΔSB) showed a diffuse cytosolic localization (Fig. 1*B*). Surprisingly, we found that when iNOS and SPSB1 were co-expressed, iNOS was co-localized with SPSB1 with a significant change in the subcellular distribution from dense regions to diffuse expression similar to the localization of SPSB1 (Fig. 1*C*). The subcellular redistribution of iNOS was also observed when SPSB4 was co-expressed, and, to a lesser degree, when SPSB2 was co-expressed (supplemental Fig. S2*A*). In contrast, the iNOS(N27A) mutant did not co-localize with SPSB1 and the subcellular distribution of this mutant was unchanged (Fig. 1*D*). Next, we examined whether the SOCS box was involved in the subcellular redistribution of iNOS. We found that the subcellular distribution of iNOS was unaffected by the expression of SPSB1ΔSB, despite its strong co-localization with SPSB1ΔSB (Fig. 1*E*). Instead, the subcellular distribution of SPSB1ΔSB changed substantially, from diffuse to dense regions of expression, similar to the localization of

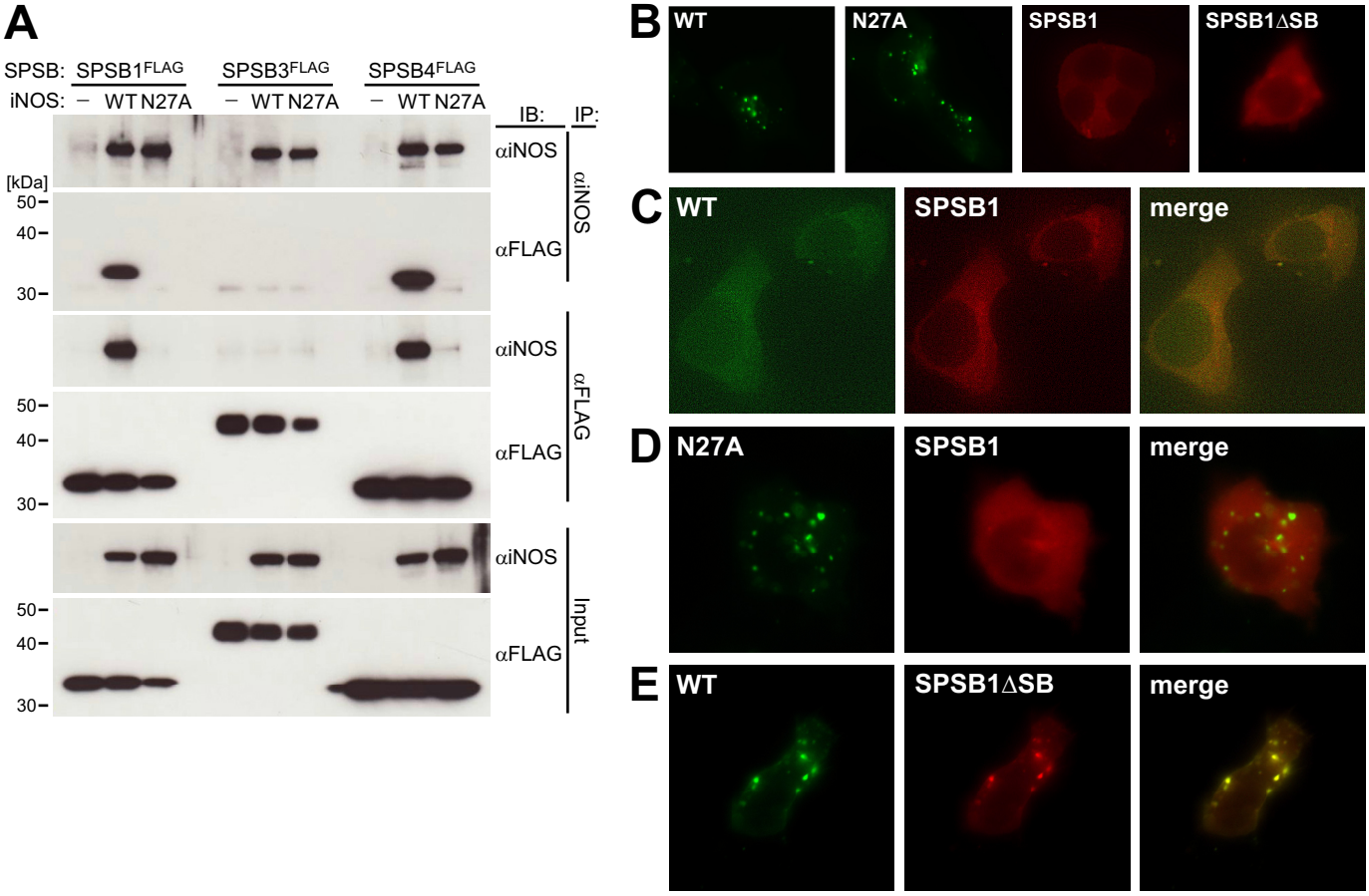

FIGURE 1. **SPSB1 and SPSB4 interact with iNOS in a D-I-N-N-N sequence-dependent manner and induce the subcellular redistribution of iNOS in a SOCS box-dependent manner.** *A*, HEK293T cells were transfected with indicated expression plasmids for 24 h. The cell lysates were prepared and subjected to immunoprecipitation and immunoblotting. *B*, in 12-well plates, HEK293T cells were transfected with an expression plasmid for iNOS-YFP, iNOS(N27A)-YFP, SPSB1-CFP, or SPSB1ΔSB-CFP for 12 h. Then the cells were placed into a 35-mm glass bottom dish. The next day, the cells were examined by fluorescence microscopy. *C–E*, HEK293T cells were transfected with expression plasmids for iNOS-YFP and SPSB1-CFP (*C*), iNOS(N27A)-YFP and SPSB1-CFP (*D*), or iNOS-YFP and SPSB1ΔSB-CFP (*E*). The cells were subjected to fluorescence microscopy as described in *B*. iNOS and iNOS(N27A) mutant are shown in *green* (pseudocolor), and SPSB1 and SPSB1ΔSB are shown in *red* (pseudocolor). Regions with co-localization appear *yellow*.

iNOS. These results suggest that an extensive subcellular redistribution of iNOS in the cytosol was caused by SPSB1 and SPSB4, and, to a lesser extent, by SPSB2, and that the SOCS box was required for this.

SPSB1 and SPSB4 Induce the Proteasomal Degradation of iNOS More Strongly than SPSB2—Although Kuang *et al.* (20) showed that SPSB2 targets iNOS for proteasomal degradation, whether SPSB1 and SPSB4 also play a role in the proteasomal degradation of iNOS remains unclear. Thus, we first examined whether iNOS levels were down-regulated by the overexpression of SPSB1 and SPSB4. We found that iNOS levels were slightly decreased when SPSB1 and SPSB4 were overexpressed, while the overexpression of SPSB2 did not affect the iNOS level (Fig. 2*A*), different from the previous observations of Kuang *et al.* We next examined whether the co-overexpression of SPSB1 and SPSB4 induced the down-regulation of iNOS more than the expression of SPSB1 or SPSB4 alone. Surprisingly, the iNOS level was unchanged in cells expressing both SPSB1 and SPSB4 compared with the control cells (Fig. 2*B*). These results led us to hypothesize that the down-regulation of iNOS is not always proportional to the expression levels of SPSB proteins. To test this, HEK293T cells in 24-well plates were transiently transfected with cDNAs expressing iNOS (200 ng) and each SPSB protein (0.01–600 ng) for 24 h, and then iNOS levels in the cells and nitrite accumulation in the cell culture medium were analyzed by immunoblotting and the Griess reaction assay, respectively. We found that both the iNOS level and nitrite accumulation decreased substantially when SPSB1 and SPSB4 were expressed by transfection with 200:1 to 200:10 ng cDNA ratios of iNOS to SPSB (Fig. 2, *C* and *D*). This effect was also observed, to a lesser degree, when SPSB2 was expressed. Surprisingly, this effect was diminished when the expression level of SPSB1 and SPSB4 was increased by transfection with 200:100 to 200:600 ng cDNA ratios of iNOS to SPSB. Additionally, this effect was completely abrogated when the expression level of SPSB2 was increased. Consistent with the degradation of iNOS, the much lower expression of SPSB2 induced the subcellular redistribution of iNOS (supplemental Fig. S2*B*). These results suggest that the molar ratio of iNOS to SPSB is important for iNOS regulation, and that SPSB1 and SPSB4 have a more potent effect on the subcellular redistribution and down-regulation of iNOS than SPSB2.

Next, to determine whether the down-regulation of iNOS was caused by accelerated degradation of iNOS, we examined

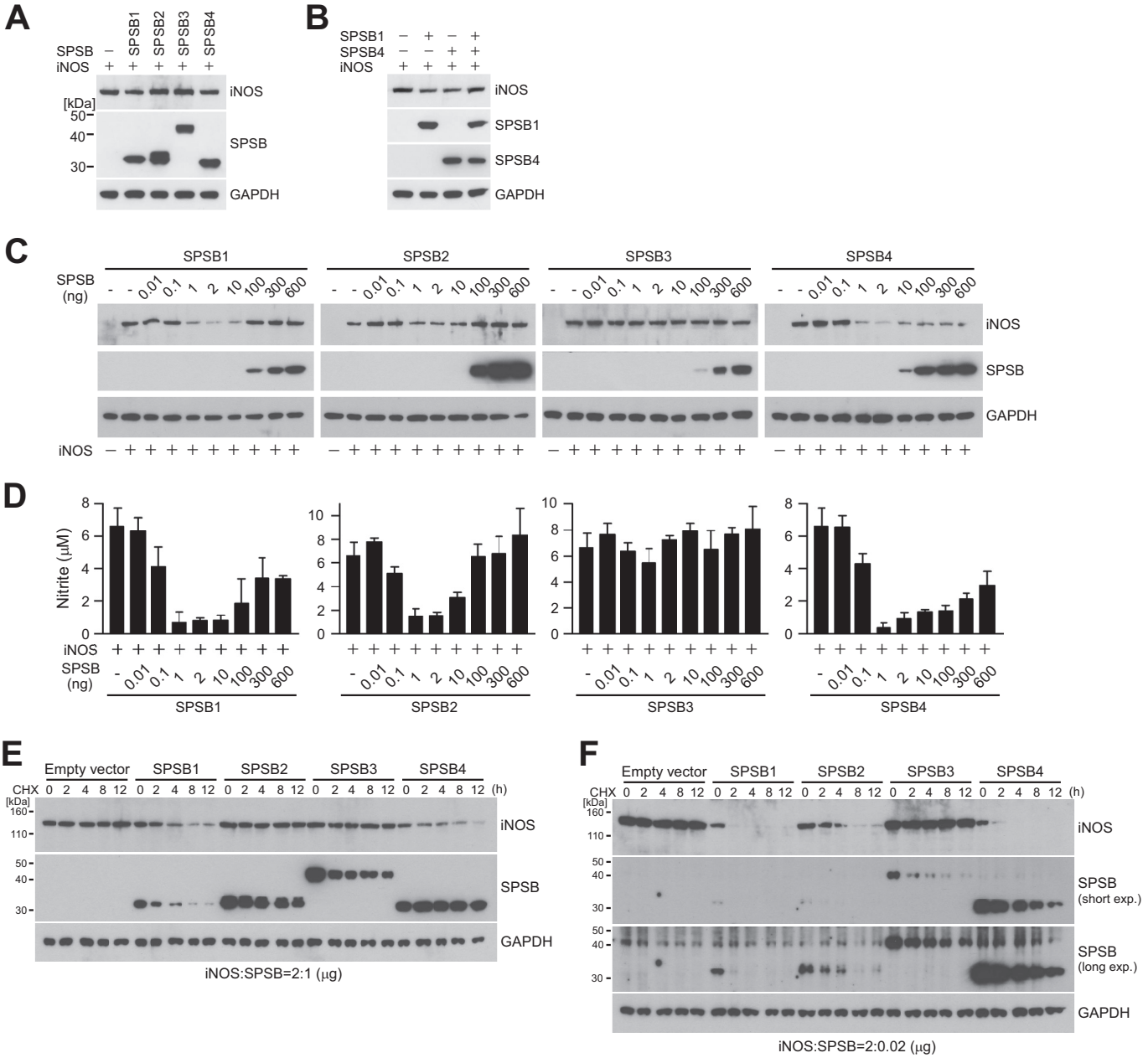

FIGURE 2. **SPSB1 and SPSB4 induce the degradation of iNOS more strongly than SPSB2.** *A*, HEK293T cells in 12-well plates were transfected with expression plasmids for iNOS (0.8 μg), FLAG-tagged SPSBs (0.2 μg), and the pEFBOSEX vector (0.6 μg) for 24 h. Then, the cell lysates were prepared, and subjected to immunoblotting. *B*, HEK293T cells in 12-well plates were transfected with expression plasmids for iNOS (0.8 μg), FLAG-tagged SPSB1 (0.2 μg), and/or HA-tagged SPSB4 (0.2 μg) for 24 h. The pEFBOSEX vector was also transfected to ensure that a total of 1.6 μg of DNA was used per transfection. Then, the cell lysates were prepared, and subjected to immunoblotting. *C* and *D*, HEK293T cells in 24-well plates were transfected with expression plasmids for iNOS (200 ng) and FLAG-tagged SPSBs (0.01–600 ng) for 24 h. The pEFBOSEX vector was also transfected to ensure that a total of 800 ng of DNA was used per transfection. Then, the cell lysates were prepared, and subjected to immunoblotting (*C*). In addition, concentration of nitrite in the cell culture medium was assessed by the Griess assay (*D*). Data represent mean ± S.D., $n = 3$. *E* and *F*, HEK293T cells in 6-well plates were transfected with expression plasmids for iNOS and FLAG-tagged SPSBs at the indicated ratio of iNOS to SPSBs for 12 h. Then the cells were placed into 5 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.

the stability of iNOS protein in cycloheximide (CHX) chase assays (27). We found that SPSB1 and SPSB4, but not SPSB2, triggered iNOS degradation in cells transfected with cDNAs at a 2:1 μg ratio of iNOS to SPSB (Fig. 2*E*). Consistent with the results in Fig. 2, *C* and *D*, the much lower expression of SPSB1 and SPSB4 by transfection with cDNAs at a 2:0.02 μg ratio of iNOS to SPSB induced more rapid degradation of iNOS (Fig. 2*F*) that was abolished in cells treated with the proteasome inhibitor MG-132 (supplemental Fig. S3), indicating that both SPSB1 and SPSB4 target iNOS for proteasomal degradation. The lower expression of SPSB2 also triggered iNOS degradation, but this degradation was much weaker than that mediated by SPSB1 and SPSB4.

To determine whether the physical interaction between iNOS and SPSB proteins is necessary for the down-regulation of iNOS, we performed the same experiments using the iNOS(N27A) mutant. We found that, in contrast to wild-type iNOS, the levels of the iNOS(N27A) mutant and NO produc-

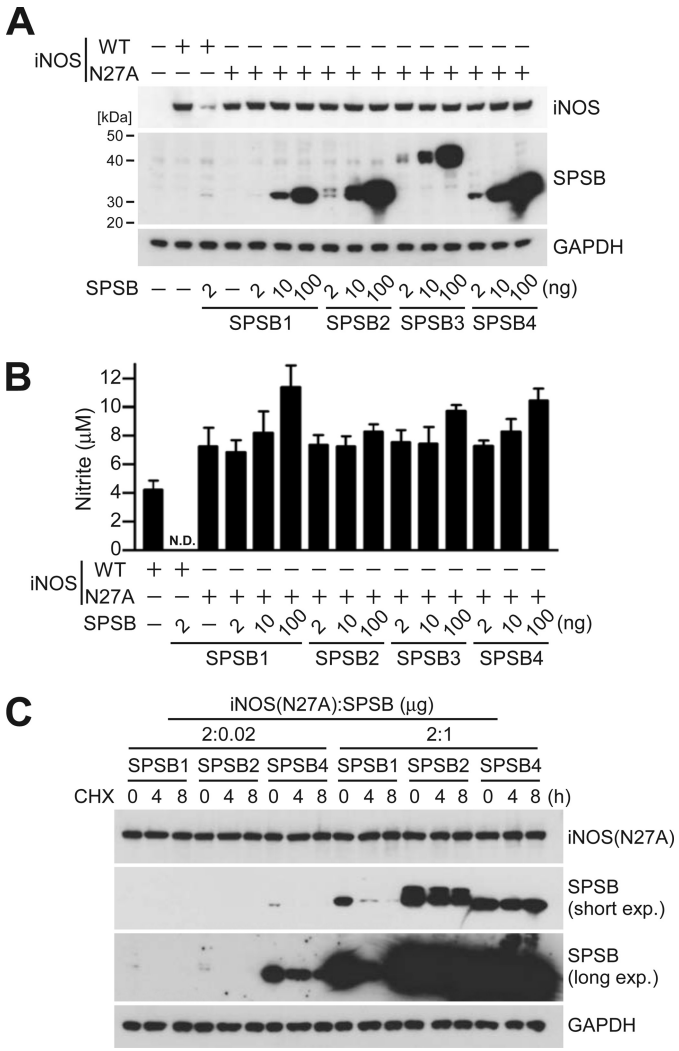

FIGURE 3. **The iNOS(N27A) mutant is completely resistant to protein degradation mediated by SPSB1, SPSB2, and SPSB4.** *A* and *B*, HEK293T cells in 24-well plates were transfected with expression plasmids for the iNOS(N27A) mutant (200 ng) and FLAG-tagged SPSBs (2–100 ng) for 24 h. The pEFBOSEX vector was also transfected to ensure that a total of 800 ng of DNA was used per transfection. Then, the levels of the iNOS(N27A) mutant and concentration of nitrite in the cell culture medium were determined as described in the legend to Fig. 2, *C* and *D*. Data represent mean ± S.D., $n = 3$. *C*, HEK293T cells in 6-well plates were transfected with expression plasmids for the iNOS(N27A) mutant and FLAG-tagged SPSBs at the indicated ratio of the iNOS(N27A) mutant to SPSBs for 12 h. Then the cells were placed into 3 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.

tion via this mutant were not affected by the expression of any of the SPSB proteins (Fig. 3, *A* and *B*). Additionally, the lifetime of the iNOS(N27A) mutant was unchanged by SPSB1, SPSB2, and SPSB4 (Fig. 3*C*), demonstrating that the iNOS(N27A) mutant is resistant to protein degradation mediated by these SPSB proteins.

SPSB1 and SPSB4 Induce the Ubiquitination of iNOS More Potently than SPSB2—Because SPSB1 and SPSB4 have more potent effects on the proteasomal degradation of iNOS than SPSB2, we next investigated the relative contributions of SPSB1, SPSB2, and SPSB4 to iNOS ubiquitination. To evaluate the level of ubiquitinated iNOS, HEK293T cells stably expressing Myc-tagged ubiquitin were transfected with cDNAs expressing iNOS and SPSB proteins for 24 h, followed by treatment with MG-132 for 4 h to accumulate ubiquitinated proteins. iNOS was immunoprecipitated with an iNOS antibody, and then the levels of ubiquitinated iNOS were analyzed by immunoblotting using a Myc antibody. We confirmed the expression of transfected iNOS and SPSB proteins by immunoblotting (Fig. 4*A*). We found that the level of ubiquitinated iNOS was enhanced, 4.6-, 2.9-, and 5.6-fold in cells transfected with SPSB1, SPSB2, and SPSB4, respectively (Fig. 4, *B* and *C*). These results indicate that, consistent with the degradation of iNOS, SPSB1 and SPSB4 induce the ubiquitination of iNOS more potently than SPSB2.

SPSB1, SPSB2, and SPSB4 Form a Bridge between iNOS and the Elongin C-Cul5 E3 Ubiquitin Ligase Complex—It has been shown that SOCS box-containing proteins interact with Elongin C and Cul5, components of the ECS E3 ubiquitin ligase complex (7), suggesting that SOCS box-containing proteins may be substrate recognition subunits of the ECS E3 ubiquitin ligase complex that polyubiquitinates substrate proteins, targeting them for proteasomal degradation. However, no direct evidence indicates that SPSB proteins mediate the interaction between the substrate and the ECS E3 ubiquitin ligase complex. Thus, we examined whether SPSB1, SPSB2, and SPSB4 mediate the iNOS interactions with Elongin C and Cul5. HEK293T cells stably expressing 3×T7-tagged Elongin C were transfected with cDNAs expressing iNOS and SPSB proteins. iNOS was immunoprecipitated with an iNOS antibody, and then the interaction with iNOS was analyzed by immunoblotting. We confirmed the expression of transfected iNOS, SPSB, and Elongin C by immunoblotting (Fig. 5*A*). We found that both Elongin C and endogenous Cul5 were co-immunoprecipitated with iNOS when SPSB1, SPSB2, and SPSB4 were expressed (Fig. 5*B*). In contrast, SPSB3 and SPSBΔSB mutants could not mediate the iNOS interactions with Elongin C and Cul5, probably because of their respective inability to interact with iNOS and Elongin C/Cul5. Next, we immunoprecipitated Elongin C with a T7 antibody and analyzed the interaction of iNOS with Elongin C by immunoblotting. We found that iNOS was co-immunoprecipitated with Elongin C when SPSB1, SPSB2, and SPSB4 were expressed (Fig. 5*C*). In contrast, SPSB3 could not mediate an interaction between Elongin C and iNOS, although SPSB3 was co-immunoprecipitated with Elongin C. The SPSBΔSB mutants were not co-immunoprecipitated with Elongin C, because they lack a SOCS box. These results demonstrate that SPSB1, SPSB2, and SPSB4 form a bridge between iNOS and the ECS E3 ubiquitin ligase complex in a SOCS box-dependent manner.

The N-terminal Fragment of iNOS Could Inhibit iNOS Degradation by Disrupting iNOS-SPSB Interactions—Cell and tissue microarray expression data mined from the Genomic Institute of the Novartis Research Foundation (GNF) indicated that SPSB1, SPSB2, and SPSB4 mRNAs are broadly expressed in various murine cell types and tissues (supplemental Fig. S4). We also confirmed by reverse transcription-polymerase chain reaction (RT-PCR) that both SPSB1 and SPSB2 mRNAs were expressed in cells of the mouse macrophage cell line RAW264.7 (supplemental Fig. S5). These results suggest that SPSB1, SPSB2, and SPSB4 may compensate for each of the other's loss of function if the expression of a certain SPSB is lost, as a result

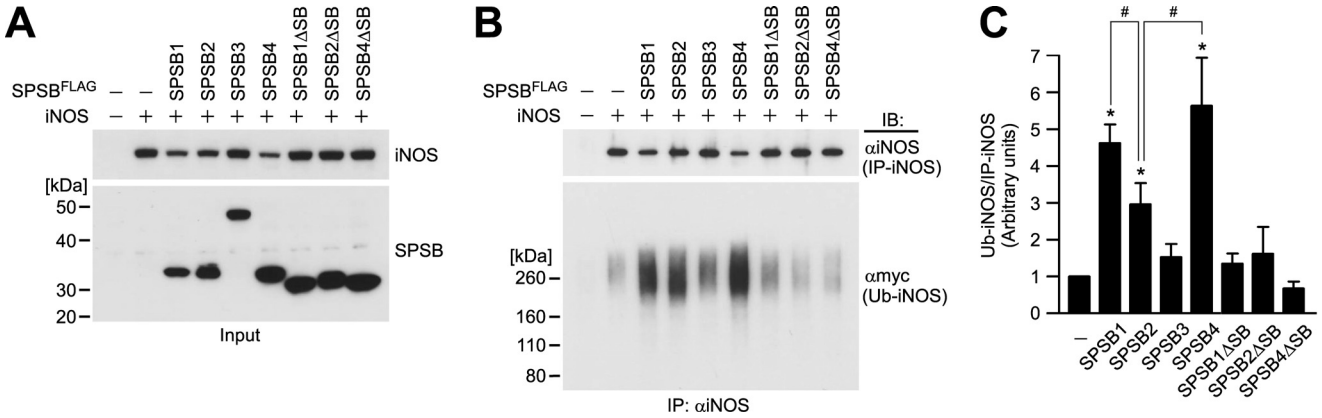

FIGURE 4. **SPSB1 and SPSB4 induce the ubiquitination of iNOS more potently than SPSB2.** *A* and *B*, In 6-well plates, HEK293T cells stably expressing Myc-tagged ubiquitin were transfected with expression plasmids for iNOS (2 μg) and FLAG-tagged SPSB (20 ng) for 24 h followed by treatment with 5 μM MG-132 for 4 h. Part of each cell lysate was subjected to immunoblotting (*A*). The remaining part of each 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*). *C*, quantification of the levels of ubiquitinated iNOS shown in *B*. Data represent mean ± S.D., $n = 3$. *, $p < 0.05$, compared with samples from cells not transfected with SPSB. #, $p < 0.05$, compared with samples from cells transfected with SPSB2.

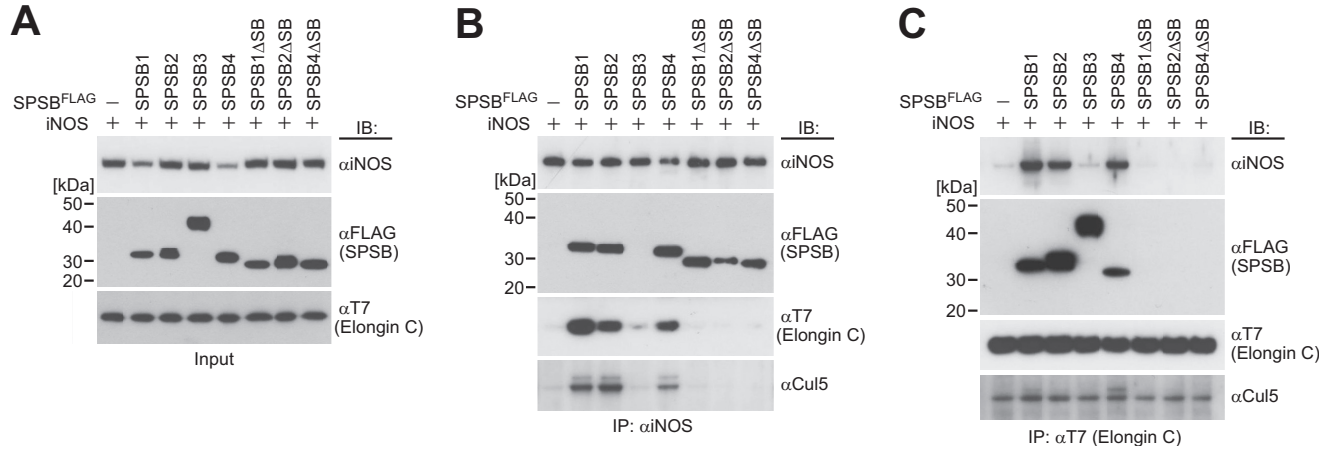

FIGURE 5. **iNOS binds to Elongin C and Cul5 via SPSB1, SPSB2, and SPSB4.** In 6-well plates, HEK293T cells stably expressing 3xT7-tagged Elongin C were transfected with expression plasmids for iNOS (2 μg) and FLAG-tagged SPSB (0.5 μg) for 24 h. The cell lysates were prepared and part of each cell lysate was subjected to immunoblotting (*A*). The remaining part of each cell lysate was subjected to immunoprecipitation using an anti-iNOS antibody (*B*) and an anti-T7 antibody (*C*). Then, immunoblotting was carried out using the indicated antibodies.

of siRNA or gene knock-out. To investigate the biochemical and physiological significance of SPSB1, SPSB2, and SPSB4 during iNOS induction in macrophages, we attempted to disrupt iNOS-SPSB interactions by overexpression of the iNOS N-terminal fragments containing a D-I-N-N-N motif, because 1) the interaction between iNOS and SPSB proteins is crucial for iNOS degradation, 2) similar to full-length iNOS, the iNOS N-terminal fragment, such as residues 1–124 of human iNOS, could interact with SPSB1, SPSB2, and SPSB4, and thus would both compete with full-length iNOS for interaction with these SPSB proteins to prevent the degradation of iNOS, and 3) it is much easier to express iNOS N-terminal fragments in macrophages than to introduce siRNAs against multiple gene targets into cells or to generate multiple gene knock-out mice. As expected, the overexpression of 1–124 iNOS fragments disrupted iNOS interactions with SPSB1, SPSB2, and SPSB4 (Fig. 6*A*) and prevented both iNOS degradation and a decrease in iNOS-mediated NO production (Fig. 6, *B* and *C*). In contrast, the 1–124 iNOS fragments carrying the N27A mutation did not alter the down-regulation of iNOS and a decrease in NO production mediated by SPSB1, SPSB2, and SPSB4 (Fig. 6, *D* and *E*, and data not shown). These results indicate that the iNOS N-terminal fragment acted as an inhibitor of SPSB1, SPSB2, and SPSB4.

Disruption of iNOS-SPSB Interactions Attenuates iNOS Ubiquitination and Prolongs the Lifetime of iNOS in Lipopolysaccharide (LPS)-activated Macrophages—To determine the biochemical and physiological significance of SPSB1, SPSB2, and SPSB4 during iNOS induction in macrophages, we established RAW-TetOFF-miNOS(1–118)^{FLAG} cells in which the mouse iNOS N-terminal fragment (residues 1–118) was reversibly expressed by a tetracycline-inducible system (residues 1–118 of mouse iNOS correspond to residues 1–124 of human iNOS). We found that the iNOS level reached its peak within 12 h of LPS stimulation and was sustained for up to at least 48 h in cells expressing the 1–118 iNOS fragments, whereas the iNOS level in the control cells reached its peak within 6 h of LPS stimulation and then declined (Fig. 7*A*). We also examined the levels of COX-2, JAK-2, and GAPDH as controls. COX-2 is an LPS-inducible protein similar to iNOS, JAK-2 is degraded

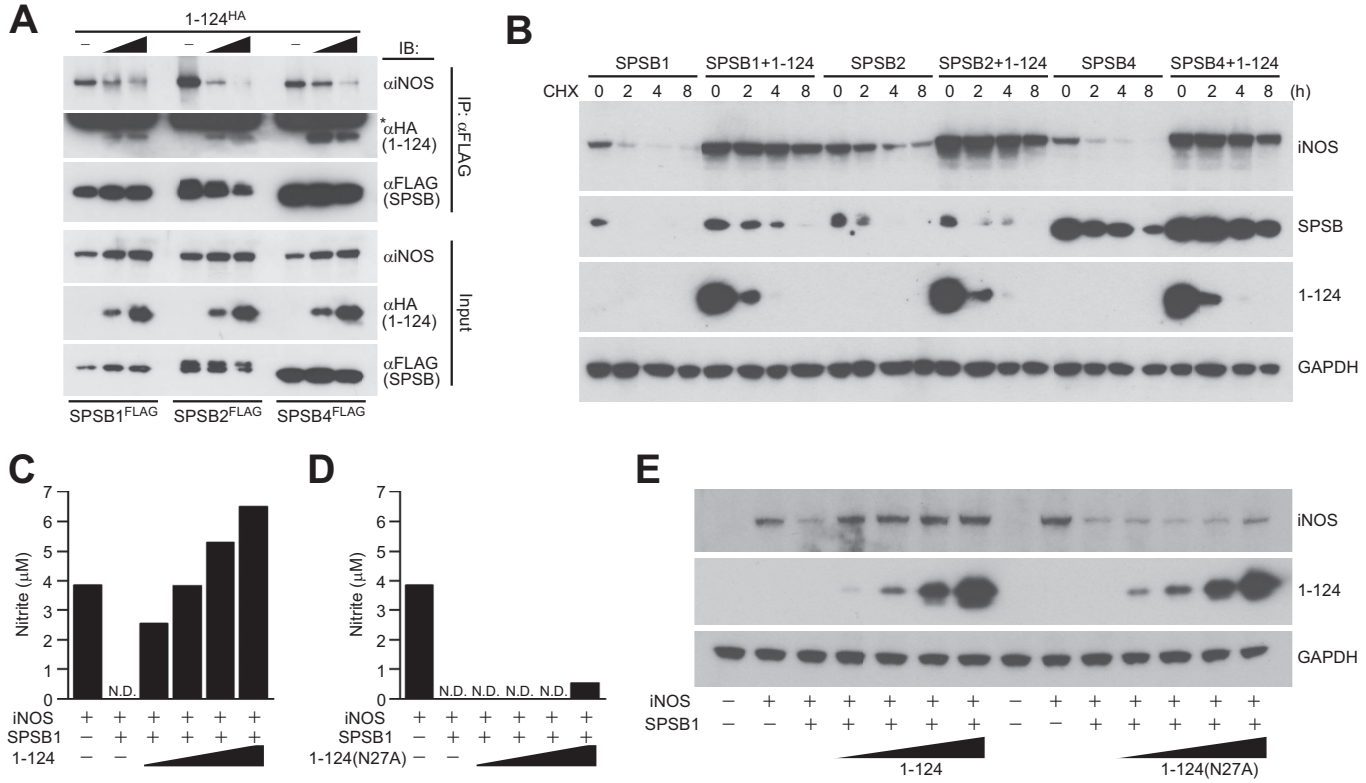

FIGURE 6. **The iNOS N-terminal fragment acts as an inhibitor of SPSB1, SPSB2, and SPSB4.** *A*, HEK293T cells in 6-well plates were transfected with expression plasmids for iNOS (1 μg), FLAG-tagged SPSB (20 ng), and HA-tagged 1–124 iNOS fragment (0, 1, and 3 μg) for 24 h followed by treatment with 5 μM MG-132 for 4 h. The cell lysates were prepared and subjected to immunoprecipitation and immunoblotting. A star indicates the light chain of the antibody. *B*, HEK293T cells in 6-well plates were transfected with expression plasmids for iNOS (2 μg), FLAG-tagged SPSB (20 ng), and HA-tagged 1–124 iNOS fragment (2 μg) for 12 h. Then 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. *A*∼*B*, the pEFBOSEX vector was also transfected to ensure that a total of 4 μg of DNA was used per transfection. *C*∼*E*, HEK293T cells in 24-well plates were transfected with expression plasmids for iNOS (200 ng), a FLAG-tagged SPSB (2 ng), and either an HA-tagged 1–124 iNOS fragment or an HA-tagged 1–124(N27A) iNOS fragment (50, 100, 200, and 600 ng) for 24 h. Then nitrite accumulation in the cell culture medium was assessed by the Griess assay (*C* and *D*). *N.D.*: not detected. The cell lysates were prepared from the cells shown in *C* and *D*, and were subjected to immunoblotting (*E*). *C*∼*E*, pEFBOSEX vector was also transfected to ensure that a total of 800 ng of DNA was used per transfection.

under the control of another SOCS box protein, SOCS1 (28), and GAPDH is a typical internal control for immunoblotting. The kinetics of expression for each control protein were almost identical in the absence and presence of the 1–118 iNOS fragment. Consistent with sustained iNOS levels, nitrite accumulation in response to LPS stimulation was substantially elevated in the cell culture medium of cells expressing the 1–118 fragments (Fig. 7*B*). Next, we performed CHX chase assays to examine the lifetime of iNOS in LPS-activated RAW264.7 macrophages. We found that the lifetime of iNOS in cells expressing the 1–118 fragments was markedly prolonged, whereas the lifetime of control proteins was unchanged (Fig. 7*C*). We further examined the level of ubiquitinated iNOS 12 h after LPS stimulation of RAW264.7 macrophages. We found that the level of ubiquitinated iNOS was greatly diminished by the expression of the 1–118 fragments (Fig. 7, *D* and *E*). We confirmed the specificity of the 1–118 iNOS fragment to SPSB proteins and the inhibitory effect of this fragment on SPSB proteins using cells expressing the 1–118 iNOS fragment carrying the N27A mutation (1–118(N27A); supplemental Fig. S6, *A*–*E*). These results demonstrate that SPSB1, SPSB2, and SPSB4 negatively regulate the lifetime of iNOS by promoting its ubiquitination and proteasomal degradation in LPS-activated macrophages.

SPSB1, SPSB2, and SPSB4 Prevent the Cytotoxic Effect of iNOS in LPS-activated Macrophages—Because excessive amounts of NO produced via iNOS can result in cytotoxicity and apoptosis mediated by NO reactions with proteins and nucleic acids (29), we assessed NO-induced cell death 48 h after LPS stimulation of RAW264.7 macrophages. We found that nearly 70% of RAW264.7 cells expressing the 1–118 fragments were 7-AAD positive cells (dead cells), but this cytotoxic effect was almost completely abolished in cells treated with the iNOS inhibitor 1400W (30) (Fig. 7, *F* and *G*), suggesting that cell death was caused by iNOS-derived NO. In contrast, despite LPS stimulation, NO-induced cytotoxicity was not observed in control cells and cells expressing the 1–118(N27A) fragments (supplemental Fig. S6, *F* and *G*). These results indicate that SPSB1, SPSB2, and SPSB4 play an essential role in protection against the cytotoxic effect of iNOS in LPS-activated macrophages.

DISCUSSION

The three NOS isoforms have several distinct characteristics, some of which depend on the N-terminal short region located before the oxygenase domain, because this region contains a domain or motif that is unique to each NOS isoform and thus endows each NOS isoform with specific biochemical and phys-

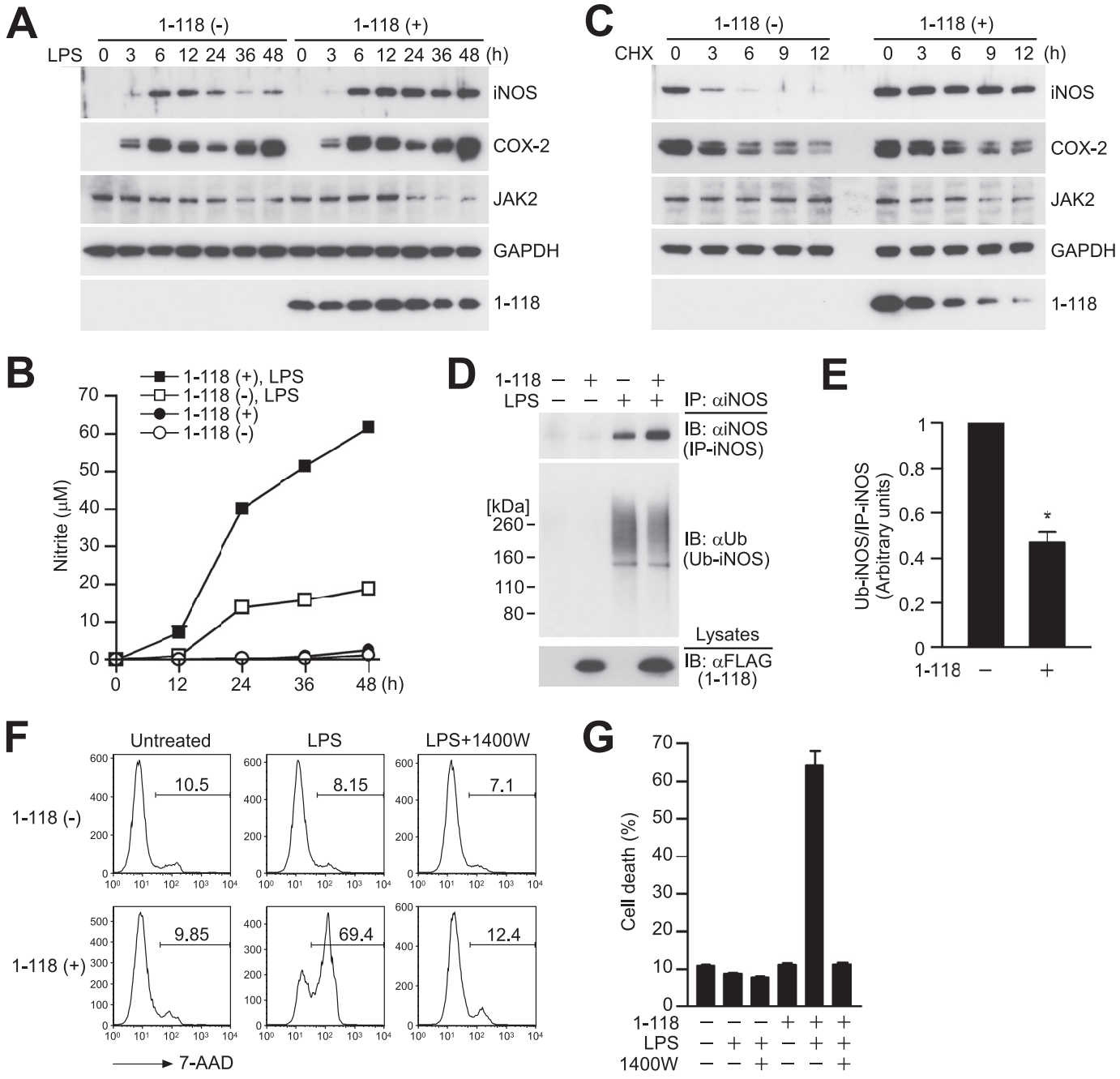

FIGURE 7. **The biological and physiological significance of SPSB1, SPSB2, and SPSB4 during iNOS induction in LPS-activated RAW264.7 macrophages.** *A*, 1–118 iNOS fragment was expressed by removing doxycycline (*DOX*) from the culture media of RAW-TetOFF-miNOS(1–118)^{FLAG} cells for 24 h. Then the cells were stimulated with 10 ng/ml LPS for the indicated periods. The cell lysates were prepared and subjected to immunoblotting. *B*, cells were treated as described in *A*, and cell culture supernatants were collected at the indicated time points. Then nitrite accumulation in the supernatants was assessed by the Griess assay. *C*, 1–118 iNOS fragment was expressed as described in *A*. Then the cells were stimulated with 10 ng/ml LPS for 12 h followed by treatment with 100 μM CHX for the indicated periods. The cell lysates were prepared and subjected to immunoblotting. *D*, 1–118 iNOS fragment was expressed as described in *A*. Then the cells were stimulated with 10 ng/ml LPS for 12 h followed by treatment with 5 μM MG-132 for 4 h. The cell lysates were prepared and subjected to immunoprecipitation using an anti-iNOS antibody. Immunoprecipitants were then subjected to immunoblotting with an anti-ubiquitin antibody to visualize the ubiquitinated iNOS. *E*, level of ubiquitinated iNOS shown in *D* was quantified as a ratio of the density of anti-ubiquitin immunoblotting (Ub-iNOS) *versus* anti-iNOS immunoblotting (IP-iNOS). Data represent mean ± S.D., $n = 3$. *, $p < 0.05$. *F*, 1–118 iNOS fragment was expressed as described in *A*. Then the cells were treated with 10 ng/ml LPS with or without 100 μM 1400W for 48 h. Cells were collected and cell viability was analyzed by 7-AAD staining. *G*, quantification of cell viability shown in *F*. Data represent mean ± S.D., $n = 3$.

iological features (21, 22). Here, we present studies examining the intrinsic mechanisms of iNOS regulation by identifying and characterizing proteins that interact specifically with the N-terminal region of iNOS. Our results reveal that SPSB1, SPSB2, and SPSB4 interact with the N-terminal region of iNOS, induce the subcellular redistribution of iNOS, and form a bridge between iNOS and the ECS E3 ubiquitin ligase complex to target iNOS for ubiquitin/proteasome-dependent degradation. SPSB1 and SPSB4 have greater effects on these biological processes of iNOS regulation than SPSB2. The negative regulation of iNOS by these SPSB proteins contributes importantly to protection against the cytotoxic effect of iNOS in activated macrophages.

Although more than 50 SOCS box-containing proteins have been found and are thought to act as substrate recognition subunits of the ECS E3 ubiquitin ligase complex, only a few substrates for SOCS box-containing proteins have been identified. Our observation that iNOS was clearly immunoprecipitated with Elongin C and Cul5 in the presence of SPSB1, SPSB2, and SPSB4 is the first direct evidence that the SPSB family of proteins links the substrate with the ECS E3 ubiquitin ligase complex. The bioinformatic analysis by Kuang *et al.* (20) reported that 11 mouse and 16 human proteins contained the (D/E)-(I/L)-N-N-N sequence, suggesting that those proteins may also be regulated by SPSB1, SPSB2, and SPSB4. Future analysis of those proteins using the experimental systems described here may identify new substrates of SPSB1, SPSB2, and SPSB4, and contribute significantly to understanding the physiological roles of these SPSB proteins.

The few studies that have investigated the subcellular localization of iNOS have provided diverse findings. iNOS has been reported to reside in cells as a diffuse cytosolic protein (31) and vesicles of 50–80 nm in size (32) or to be localized in the perinuclear region (33) and Golgi apparatus (31). It has recently been reported that iNOS forms the aggresome with Hsc70, the C terminus of Hsp70-interacting proteins (CHIP), histone deacetylase 6, and dynein (34). Our results showed that the vesicle/aggresome-like localization of iNOS-YFP proteins was changed dramatically to a diffuse cytosolic localization by SPSB1, SPSB2, and SPSB4 (Fig. 1*C* and supplemental Fig. S2). SPSB1, SPSB2, and SPSB4 may access iNOS in the vesicles/aggresomes to direct the subcellular redistribution of iNOS that may be dependent on the subcellular localization of Elongin C and/or Cul5 because the SPSB1ΔSB mutant could not induce the subcellular redistribution and ubiquitin/proteasome-dependent degradation of iNOS despite strong co-localization with iNOS. The morphological patterns of iNOS localization may also be dependent on the expression levels of iNOS and SPSB proteins in each cell type, because the molar ratio of iNOS to SPSB is important for iNOS regulation (Fig. 2 and supplemental Fig. S2*B*).

We consistently observed a weak effect of SPSB2 on iNOS regulation regarding the subcellular redistribution and ubiquitin/proteasome-dependent degradation compared with the effects of SPSB1 and SPSB4. A binding affinity analysis using the SPRY domains of human SPSB1–4 together with human Par-4 peptide recently demonstrated that the binding affinity of SPSB2 to Par-4 was far inferior to those of SPSB1 and SPSB4 (35). Consistent with this, we found that despite the highest co-immunoprecipitation efficiency of SPSB2 with Elongin C, iNOS was co-immunoprecipitated less with Elongin C in cells expressing SPSB2 than in cells expressing SPSB1 and SPSB4 (Fig. 5*C*), suggesting that the binding affinity of SPSB2 to iNOS might also be weaker than those of SPSB1 and SPSB4. The detailed mechanism responsible for the weak binding affinity of SPSB2 is not clear presently, but the cause may be the lower sequence conservation of SPSB2 relative to SPSB1 and SPSB4.

Intriguingly, iNOS was significantly degraded in the presence of quite low levels of SPSB proteins, but this effect was diminished when the expression level of SPSB proteins was increased. These results indicate that iNOS degradation is not always proportional to the expression level of SPSB proteins. Unlike the single molecule E3 ubiquitin ligase, the activity of the multiprotein E3 ubiquitin ligase complex is likely to be affected by the expression level of each component, especially the substrate recognition subunit, as with SPSB proteins. This is because in the presence of excessive levels of SPSB proteins, Elongin C and Cul5 may be saturated by SPSB proteins, and free SPSB proteins may capture the substrate before the substrate interacts with the ECS-SPSB complex, and eventually SPSB proteins stabilize the substrate. Indeed, we observed that the iNOS level was significantly increased when SPSB2 was overexpressed in LPS-activated RAW264.7 macrophages (data not shown). Interestingly, mRNA expression data from GNF indicated that the mRNA expression levels of SPSB2 and SPSB4 were decreased markedly after stimulation with LPS in both bone marrow-derived macrophages and thioglycolate-elicited peritoneal macrophages (supplemental Fig. S4). Additionally, Kuang *et al.* (20) found that SPSB2 mRNA was greatly downregulated after LPS+IFN-γ treatment in bone marrow-derived macrophages. Thus, our results and those observations suggest that LPS(+IFN-γ)-induced down-regulation of SPSB proteins might facilitate the proteasomal degradation of iNOS in cells expressing high levels of SPSB proteins under basal conditions.

We still detected ubiquitinated iNOS in LPS-activated RAW264.7 macrophages expressing iNOS N-terminal fragments, although it was less than half of that observed in control cells (Fig. 7, *D* and *E*). The causes for this observation may be that the iNOS N-terminal fragments could not completely disrupt iNOS-SPSB interactions, and that other E3 ubiquitin ligases are involved in the proteasomal degradation of iNOS. Regarding the latter, it has recently been reported that CHIP, a U-box-type E3 ubiquitin ligase (36), promotes the proteasomal degradation of iNOS (34, 37). Unlike the direct interaction between iNOS and SPSB proteins, the interaction between iNOS and CHIP is mediated by Hsp70 (34), not the D-I-N-N-N sequence, suggesting that CHIP can interact with the iNOS(N27A) mutant as well as wild-type iNOS, and mediate the proteasomal degradation of this mutant. Because the iNOS(N27A) mutant is completely resistant to protein degradation mediated by ECS(SPSB1/2/4), the extent to which other E3 ubiquitin ligases, such as CHIP, are involved in the proteasomal degradation of iNOS could be determined by the degree of degradation of the iNOS(N27A) mutant. Future studies using iNOS(N27A) knock-in mice will provide important insight not only into the physiological significance of the ECS(SPSB1/2/4) E3 ubiquitin ligase complexes during iNOS induction, but also the contribution of other E3 ubiquitin ligases to the proteasomal degradation of iNOS in each cell type and tissue.

NO production by iNOS in activated macrophages is considered to be essential for various bactericidal and tumoricidal functions (38, 39). Macrophages themselves, however, are also a target of NO by an autocrine action. Indeed, excessive NO production via iNOS has been shown to induce apoptotic cell death of activated macrophages (40–42), which may allow some bacteria to survive in the host and exacerbate inflammation at the infection site (43). In RAW264.7 macrophages, LPS stimulation did not induce cell death for up to at least 48 h, whereas in

RAW264.7 macrophages expressing the iNOS N-terminal fragments to disrupt iNOS-SPSB interactions, LPS stimulation caused substantial NO-dependent cell death (Fig. 7, *F* and *G*), demonstrating the intrinsic mechanism mediated by SPSB proteins for protection against the cytotoxic effect of iNOS in activated macrophages. Clearly, much remains to be learned regarding how SPSB proteins regulate the balance of bactericidal and tumoricidal effects *versus* the cytotoxic effect of iNOS in activated macrophages.

In summary, our results indicate that iNOS is a common substrate for SPSB1, SPSB2, and SPSB4, all of which link iNOS and the ECS E3 ubiquitin ligase complex. SPSB1 and SPSB4 have more potent effects on various biological processes of iNOS regulation than SPSB2. SPSB1, SPSB2, and SPSB4 negatively regulate NO production and limit cellular toxicity through rapid ubiquitination and proteasomal degradation of iNOS in activated macrophages. A better understanding of the physiological significance of iNOS regulation by these SPSB proteins could lead to new therapeutic strategies for many disorders in which iNOS-derived excessive NO is implicated.

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