Ubiquitination-mediated Regulation of Biosynthesis of the Adhesion Receptor SHPS-1 in Response to Endoplasmic Reticulum Stress*

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Misfolding of proteins during endoplasmic reticulum (ER) stress results in the formation of cytotoxic aggregates. The ER-associated degradation pathway counteracts such aggregation through the elimination of misfolded proteins by the ubiquitin-proteasome system. We now show that SHP substrate-1 (SHPS-1), a transmembrane glycoprotein that regulates cytoskeletal reorganization and cell-cell communication, is a physiological substrate for the Skp1-Cullin1-NFB42-Rbx1 (SCF^NFB42) E3 ubiquitin ligase, a proposed mediator of ER-associated degradation. SCF^NFB42 mediated the polyubiquitination of immature SHPS-1 and its degradation by the proteasome. Ectopic expression of NFB42 both suppressed the formation of aggregosome-like structures and the phosphorylation of the translational regulator eIF2α induced by overproduction of SHPS-1 as well as increased the amount of mature SHPS-1 at the cell surface. An NFB42 mutant lacking the F box domain had no such effects. Our results suggest that SCF^NFB42 regulates SHPS-1 biosynthesis in response to ER stress.

Adhesion molecules on the surface of cells play pivotal roles in many physiological processes, including cell growth, differentiation, and migration. Dysregulation of specific adhesion molecules has thus been shown to result in pathological conditions such as inflammation, neurodegeneration, and cancer metastasis. We previously identified SHP substrate-1 (SHPS-1) (1, 2), an adhesion receptor of the immunoglobulin superfamily, which associates with the ligand CD47 (7). Through its interaction with CD47, SHPS-1 contributes to the phagocytosis of red blood cells by macrophages (8), to macrophage multinucleation (9), to T cell activation (10), and to neutrophil transmigration (11).

The cytoplasmic region of SHPS-1 acts as a scaffold for the assembly of multiprotein complexes (1, 3, 12). By recruiting the protein-tyrosine phosphatase SHP-2 to the cell membrane, SHPS-1 negatively or positively regulates intracellular signaling initiated either by tyrosine kinase-coupled receptors for growth factors or by cell adhesion to extracellular matrix proteins (13, 14). In the present study, we sought to provide insight into SHPS-1 function by identifying proteins that regulate SHPS-1 or transduce signals emanating from its cytoplasmic region. We now demonstrate a physical and functional interaction between SHPS-1 and both neural F box protein of 42 kDa (NFB42) and S phase kinase-associated protein 1 (Skp1) in mouse brain and melanocytes. NFB42 (recently renamed Fbx2) was first identified as a gene product that is highly enriched in the nervous system (19). F box proteins contain an F box domain and mediate substrate recognition by Skp1-Cullin1-F box protein-Rbx1 (SCF)-type E3 ubiquitin-protein ligase complexes (20). Ectopic expression of NFB42 was shown to inhibit the proliferation of neuroblastoma cells (19). Skp1 is an invariant component of SCF-type E3 enzymes and is thought to control the cell cycle (21). NFB42 and Skp1 are thus implicated in the control of neuronal proliferation by protein ubiquitination, although the biological roles of these proteins remain to be established. Skp1 also participates in various signal transduction pathways, including that responsible for glucose-dependent re-assembly of V-ATPase complexes (22).

We now show that NFB42 and Skp1, together with Cullin1, constitute an SCF-type E3 ubiquitin ligase (SCF^NFB42) and that this complex catalyzes the polyubiquitination of immature forms of SHPS-1. Forced expression of NFB42 resulted in elimination of misfolded SHPS-1 molecules from the endoplasmic...
regulation (ER) by the ubiquitin-proteasome proteolytic pathway, an effect that was associated with marked inhibition of the formation of cellular aggregates containing SHPS-1. Furthermore, this elimination of misfolded SHPS-1 from the ER led to substantial up-regulation of SHPS-1 expression at the cell surface. We also provide evidence that SCF<sub>NF<sub>β</sub></sub> functions to maintain ER homeostasis during cellular stress by supporting the biosynthesis of SHPS-1.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—The cDNAs encoding the SHPS-1 mutants SHPS-1–4F, in which all four tyrosine residues in the cytoplasmic region (Tyr<sup>404</sup>, Tyr<sup>432</sup>, Tyr<sup>449</sup>, and Tyr<sup>473</sup>) are replaced by phenylalanine, and SHPS-1–JM, in which two juxtamembrane lysine residues (Lys<sup>401</sup> and Lys<sup>402</sup>) are replaced by asparagine and glutamine, respectively, were generated by site-directed mutagenesis with the full-length mouse SHPS-1 cDNA (2) as a template and a Transformer Site-directed Mutagenesis kit (Clontech). The wild-type and mutant SHPS-1 cDNAs were then inserted separately into the EcoRI and NotI site of pTracer-CMV (Invitrogen). To generate a cDNA encoding SHPS-1 fused to green fluorescent protein (GFP), we performed the polymerase chain reaction (PCR) with a full-length human SHPS-1 cDNA (2) as a template, a T7 promoter–enhanced primer 5′-AAATACGCTTCTCATTACGCG 3′. The PCR product was digested with EcoRI and SalI and then inserted into pEGFP N2 (Clontech). The pSra vector encoding wild-type rat SHPS-1 was described previously (1). The pcDNA3 vectors encoding hemagglutinin epitope (HA)-tagged versions of either wild-type rat NFB42 (amino acids 2–296) or the deletion mutants NFBAP (residues 53–296), NFBAPF (residues 95–296), or NFBPP (residues 2–94) (19) were kindly provided by R. Pittman (University of Pennsylvania). To generate cDNAs encoding Myc epitope-tagged versions of wild-type NFB42 and NFBAPF, we amplified the corresponding coding regions by PCR with the full-length NFB42 cDNA as template. The PCR products were digested with EcoRI and SalI and then inserted in-frame into pcDNA3 (Invitrogen), a mammalian expression vector that had been modified to add the coding sequence for the Myc epitope to the 5′ end of the inserted cDNA. The pcDNA3 vectors encoding Myc epitope-tagged forms of mouse Skp1 and mouse Cullin1 were kindly provided by S. Hatakeyama (Kyushu University, Japan).

**Cells, Transfection, and Antibodies**—Melan-a cells were maintained in modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 12.0-tetradecanoylphorbol 13-acetate (200 ng/ml) (Sigma). Chinese hamster ovary (CHO) cells stably transfected expressing wild-type mouse SHPS-1 (CHO-mSHPS-1 cells) were established as described previously (23) and were maintained in Ham’s F-12 medium (Sigma) supplemented with 10% FBS. Transient transfection of CHO-mSHPS-1 cells (2.4 × 10<sup>6</sup> cells/35-mm dish) was performed to add the coding sequence for the Myc epitope to the 5′ end of the inserted cDNA. The pcDNA3 vectors encoding Myc epitope-tagged forms of mouse Skp1 and mouse Cullin1 were kindly provided by S. Hatakeyama (Kyushu University, Japan).

A rat monoclonal antibody (mAb) to mouse SHPS-1 (24) and rabbit polyclonal antibodies to NFB42 (19) were described previously. Mouse mAbs to human SIRPα and to Skp1 were obtained(654,111),(794,145) from Transduction Laboratories; rabbit polyclonal antibodies to SHPS-1 and to Ser<sup>94</sup>-phosphorylated eukaryotic initiation factor 2α (eIF2α) were from Upstate Biotechnology, Inc.; rabbit polyclonal antibodies to eIF2α were from Cell Signaling Technology; rabbit polyclonal antibodies to Cullin1 were from Zymed Laboratories Inc.; a rabbit mAb to ubiquitin (mAb UB-1) was from Santa Cruz Biotechnology; mouse mAb to ubiquitin (clone 7F10) was a gift from Shigeyuki Kuwahara (Kyushu University, Japan); and normal rat or mouse IgG were from Santa Cruz Biotechnology; rabbit polyclonal antibodies to Cullin1 were kindly provided by S. Hatakeyama (Kyushu University, Japan).

**Immunofluorescence Analysis**—All procedures were performed at room temperature. Cells seeded on glass coverslips were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde in PBS for 20 min, and incubated with 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 10 min. Cells were then permeabilized for 2 min in PBS containing 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA) before incubation first for 1 h in PBS containing 0.1% Triton X-100, 10% FBS, and 0.5% BSA and then for 1 h with mAb 9E10 to the Myc tag. After washing twice with PBS containing 0.5% BSA, the cells were incubated with Texas Red-conjugated secondary antibodies for 30 min, washed three times with PBS containing 0.5% BSA, and examined with a laser-scanning confocal microscope (Bio-Rad model MRC-1024).

**Flow Cytometry**—Detected cells (0.5 × 10<sup>5</sup> to 1 × 10<sup>6</sup>) were washed with buffer B (PBS containing 1 mM EDTA and 2% FBS) and then incubated with the mAb to mouse SHPS-1 (200 ng/ml). The stained cells were washed twice, suspended in 0.025% dimethyl sulfoxide and 0.2% ethanol, respectively, and analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data were processed with CellQuest software (BD Biosciences).

**RESULTS**

**SHPS-1 Forms a Complex with NFB42 and Skp1 in Vivo**—SHPS-1 is particularly abundant in the central nervous system (4, 6, 24). We therefore adopted an immunofluorescence approach to isolate proteins that interact with SHPS-1 in the brain. A detergent-solubilized extract of mouse brain was applied to protein G-Sepharose beads that had been coupled with a mAb that reacts with the extracellular portion of mouse SHPS-1 (24). After extensive washing of the beads, bound material was eluted, resolved by SDS-PAGE, and visualized by silver staining (Fig. 1A). Bands corresponding to polypeptides that copurified with SHPS-1 were excised from the gel and subjected to in-gel digestion with trypsin, and the resulting peptides were analyzed by tandem mass spectrometry. The peptide sequences so obtained were used to search the NCBI sequence data base with the use of Mascot software. Data base searches revealed that peptide sequences derived from proteins of ~40 and ~20 kDa were identical to sequences of rat NFB42 (AF098301) (19) and mouse Skp1 (AF083214) (25), respectively (Fig. 1A). NFB42 and Skp1 were shown previously to interact directly through the P box...
FIG. 1. Identification of NFB42 and Skp1 as SHPS-1-binding proteins. A. Mouse brain extract was subjected to immunoprecipitation (IP) with a mAb targeted to the extracellular portion of mouse SHPS-1 (αSHPS-1) or with normal rat IgG (NRG), and the resulting precipitates were subjected to SDS-PAGE on a 12.5% gel and silver staining. Peptides excised from the gel were subjected to mass spectrometric analysis for identification. The positions of SHPS-1, NFB42, and Skp1, as well as those of molecular size standards (in kilodaltons), are indicated. Determined amino acid sequences corresponding to rat NFB42 or to mouse Skp1 are shown.

B. Mouse brain extract or Melan-a cell lysate was subjected to immunoprecipitation with the mAb to mouse SHPS-1, with a mAb targeted to the cytoplasmic region of human SIRPα1, or with control rat or mouse (NMG) IgG. The immunoprecipitates were then subjected to immunoblot analysis with polyclonal antibodies to NFB42 (middle panel) or with a mAb to Skp1 (bottom panel). Mouse brain extract and Melan-a cell lysate (Input) were also subjected directly to immunoblot analysis to determine the total amounts of each protein. C. Mouse brain extract was subjected to immunoprecipitation with polyclonal antibodies to NFB42 (αNFB42) or with control rabbit IgG. The immunoprecipitates were probed with polyclonal antibodies to SHPS-1 (top panel). D. Mouse brain extract and Melan-a cell lysate (Input) were also subjected directly to immunoblot analysis to determine the total amounts of each protein.
domain of the former (19), suggesting that they associate with SHPS-1 as a complex. Immunoblot analysis confirmed that NFB42 and Skp1 associate with SHPS-1 in mouse brain extract, although Skp1 was barely detectable in immunoprecipitates prepared with a mAb that reacts with the cytoplasmic region of human SIRPα1 (Fig. 1D). Endogenous SHPS-1 was also coprecipitated with antibodies to NFB42 (Fig. 1C). Immunoblot analysis also revealed that an immortal line of mouse pigmented melanocytes, Melan-a (26), expresses SHPS-1, NFB42, and Skp1 at levels similar to those observed in mouse brain extract (Fig. 1B). A substantial proportion of endogenous NFB42 was associated with SHPS-1 in Melan-a cells, although this proportion was markedly less than that apparent in mouse brain. Skp1 interaction with SHPS-1 was not detected in these cells. The interaction between SHPS-1 and NFB42 was also demonstrated in CHO cells transiently expressing recombinant SHPS-1 and HA-tagged NFB42 (Fig. 1D); NFB42 associated preferentially with immature forms of SHPS-1 in these cells, hardly interacting at all with the mature forms (Fig. 1D).

**Structural Requirements for SHPS-1-NFB42 Interaction—** Immunoprecipitation and immunoblot analysis with a brain extract prepared from mice expressing an SHPS-1 mutant that lacks most of the cytoplasmic region (15) revealed that NFB42 and Skp1 each associated with the mutant protein, albeit to a lesser extent than with wild-type SHPS-1 (Fig. 2A), suggesting that the extracellular or membrane-proximal cytoplasmic region of SHPS-1 mediates the interaction with NFB42. We next examined the ability to interact with NFB42 of SHPS-1 mutants in which two juxtamembrane lysine residues in the cytoplasmic region were replaced by asparagine and glutamine, respectively, or in which all four cytoplasmic tyrosine residues were changed to phenylalanine. Each of these mutant proteins associated with HA-tagged NFB42 in CHO cells to an extent similar to that observed with wild-type SHPS-1 (Fig. 2B). Thus, neither the positively charged juxtamembrane region nor cytoplasmic tyrosine residues of SHPS-1 appeared to play a major role in the interaction of this protein with NFB42.

To map the SHPS-1-binding site in NFB42, we employed the following three NFB42 mutant proteins: NFBAP, which lacks the NH2-terminal PEST domain; NFBAPSF, which comprises only the PEST and F box domains; and NFBPF, which comprises the NH2-terminal PEST domain; NFBAPSF, which comprises only the PEST and F box domains (Fig. 2C). The HA-tagged versions of these mutant proteins were transiently expressed in CHO cells that stably express wild-type mouse SHPS-1 (CHO-mSHPS-1 cells) and were then tested for their ability to interact with SHPS-1. NFBAP and NFBAPSF, but not NFBPF, bound to SHPS-1 (Fig. 2D), suggesting that the COOH-terminal domain of NFB42 mediates this interaction. Consistent with previous observations (19), NFBAP, but not NFBAPSF, also bound Skp1.

**SCF**<sup>NFB42</sup> **Ubiquitin Ligase Mediates Polyubiquitination and Proteasomal Degradation of Immature SHPS-1—** SCF-type ubiquitin ligase (E3) complexes include the invariant component Skp1, an F box protein that is responsible for substrate recognition, and Cullin1, which serves as a scaffold for organization of the other subunits (27). Both NFB42 (Fig. 3A) and Skp1 (Fig. 3B) interact with Cullin1, suggesting that these three proteins constitute an SCF<sup>NFB42</sup> ubiquitin ligase. Ectopic expression of NFB42 in CHO-mSHPS-1 cells revealed polyubiquitination of SHPS-1 in the presence of the specific proteasome inhibitor MG132 (Fig. 3C). Treatment of cells with MG132 also substantially increased the amount of immature SHPS-1 associated with NFB42 (Fig. 3D). Together, these results suggested that SCF<sup>NFB42</sup> acts as an E3 that catalyzes the polyubiquitination of immature SHPS-1 and that the ubiquitinated protein is subsequently eliminated by proteasomal degradation.

**SHPS-1 Is a Substrate for ER-associated Degradation—** The possibility that SCF<sup>NFB42</sup>-mediated degradation down-regulates the biological function of SHPS-1 appeared unlikely given the lack of a positive effect of MG132 on the abundance of mature SHPS-1 (Fig. 3D). Rather, the selective association of immature forms of SHPS-1 with NFB42 suggested a role for SCF<sup>NFB42</sup> in the elimination of SHPS-1 molecules misfolded during biosynthesis. To test this possibility, we determined whether SHPS-1 serves as a substrate for ER-associated degradation (ERAD), a pathway by which misfolded proteins in the ER are translocated to the cytosol, polyubiquitinated, and degraded by the proteasome (28–30). Fluorescence microscopy of Melan-a cells expressing a fusion construct of SHPS-1 and GFP (SHPS-1-GFP) revealed that the recombinant protein was localized predominantly to both the cell surface and the perinuclear region (Fig. 4A). A small proportion of SHPS-1-GFP was also detected as small aggregates throughout the cytoplasm. Similar analysis of cells exposed to MG132 showed that most SHPS-1-GFP was present either in large aggregates adjacent to the nucleus (Fig. 4B) or in small aggregates in the cytoplasm (data not shown). The large perinuclear aggregates exhibited the characteristic features of aggresomes, cellular inclusion bodies that consist of polyubiquitinated proteins and which are formed by ERAD substrates in response to inhibition of proteasome activity (31, 32). Our results thus suggested that SHPS-1 is a substrate for the ERAD pathway.

SCF<sup>NFB42</sup> Promotes Elimination of Misfolded SHPS-1 by the ERAD Pathway—** Wild-type NFB42 was distributed diffusely in the cytosol of Melan-a cells incubated in the absence (data not shown) or presence (Fig. 4G) of MG132. Overexpression of wild-type NFB42 induced redistribution of a substantial proportion of SHPS-1-GFP to the cell surface and markedly suppressed the formation of aggresome-like bodies and cytoplasmic aggregates in cells treated with MG132 (Fig. 4C; data not shown). In contrast, expression of NFBAPSF, which does not form a functional ubiquitin ligase, promoted the formation of these structures (Fig. 4D; data not shown), indicative of a dominant negative effect of this mutant. The staining of wild-type NFB42 overlapped partially with SHPS-1-GFP fluorescence in a reticular pattern throughout the cytoplasm (Fig. 4F), consistent with colocalization of these proteins at or in the ER. However, extensive colocalization of the two proteins was not detected at the cell periphery, where mature SHPS-1 is expected to reside, even in the presence of MG132. A substantial proportion of NFBAPSF colocalized with SHPS-1-GFP in the aggresome-like bodies of MG132-treated cells (Fig. 4J).

Binding experiments in vitro with *G. nivalis* agglutinin lectin, which specifically recognizes terminal mannose residues of glycoproteins (33), revealed that recombinant SHPS-1 expressed in CHO cells exists in several distinct forms with regard to N-glycan structure (Fig. 5A, left panel). Coexpression were then subjected to immunoblot analysis with the mAb to human SIRPα1 (upper panel). Duplicate precipitates were probed with the antibodies to NFB42 (lower panel). Brain extract was also subjected directly to immunoblot analysis to determine the total amounts of each protein. D. CHO-K1 cells were subjected to transient transfection with both 2 μg of pTracerCMV encoding mouse SHPS-1 and 2 μg of pcDNA3 encoding or not HA-tagged rat NFB42. Forty-eight hours after transfection, cell lysates were prepared and subjected to immunoprecipitation with mAb 12CA5 to the HA tag, and the resulting precipitates were subjected to immunoblot analysis with polyclonal antibodies to SHPS-1 (upper panel). Duplicate precipitates were probed with antibodies to NFB42 (lower panel). Cell lysates were also similarly probed to determine the expression level of each recombinant protein. Immature and mature forms of SHPS-1 are indicated.
of wild-type NFB42 substantially reduced the binding to G. nivalis agglutinin of higher electrophoretic mobility forms of SHPS-1 (under reducing conditions) but increased that of slower migrating forms. In contrast, expression of NFB\textsubscript{H9004P/H9004F} markedly enhanced the binding to G. nivalis agglutinin of high mobility forms of SHPS-1. These high mobility forms of SHPS-1 migrated as high molecular weight complexes under nonreducing conditions, suggesting that they represent misfolded SHPS-1 oligomerized by the formation of disulfide bonds (Fig. 5A, right panel). Expression of wild-type NFB42, but not that of NFB\textsubscript{H9004A/F}, substantially inhibited the formation of high molecular weight complexes of SHPS-1 induced by exposure of cells to thapsigargin (Fig. 5B), which blocks early processing of proteins in the ER. Given that terminal mannose residues are often exposed by folding intermediates of type I glycoproteins in the ER, these results suggest that SCF\textsuperscript{NFB42} interacts selectively with misfolded forms of SHPS-1 that are generated during protein maturation and thereby directs their elimination by the ERAD pathway.

**Activation of SCF\textsuperscript{NFB42} Increases SHPS-1 Expression at the Cell Surface**—We observed that the total amount of SHPS-1 in cells was substantially increased by expression of wild-type NFB42 (Figs. 1D and 5A). Of newly synthesized SHPS-1 molecules, only those that are folded into the correct tertiary structure in the ER are likely transported to the cell surface. To explore further the biological consequences of elimination of SHPS-1 by the ERAD pathway, we determined whether the presence of NFB42 affects SHPS-1 expression at the cell surface. Flow cytometry revealed that 5–10% of CHO cells subjected to transient transfection with SHPS-1 cDNA exhibited SHPS-1 immunoreactivity at the cell surface; this ratio was not substantially affected by cotransfection of the cells with wild-type NFB42 or NFB\textsubscript{H9004A/F} cDNAs (data not shown). However, coexpression of wild-type NFB42 markedly increased the pro-
portion of cells with a relatively high level of SHPS-1 expression at the cell surface (Fig. 6A). Coexpression of NFBΔAΔF had no such effect. Coexpression of neither wild-type NFB42 nor NFBΔAΔF affected the amount of GFP synthesized as a tracer for the introduction of SHPS-1 cDNA (data not shown), suggesting that the effect of NFB42 on SHPS-1 expression was not due to variation in transfection efficiency or to a nonspecific action of the recombinant NFB42 protein.

Proteins misfolded in the ER generate a stress signal that increases the phosphorylation level of eIF2α (34, 35). Overexpression of SHPS-1, but not that of some other transmembrane glycoproteins, in Melan-a cells increased the phosphorylation of eIF2α on Ser51 (Fig. 6B; data not shown). This effect was prevented by coexpression of wild-type NFB42 but, in contrast, was enhanced by that of NFBΔAΔF (Fig. 6B). These results thus suggest that SCF<sup>NFB42</sup>-mediated elimination of misfolded SHPS-1 results in up-regulation of the surface expression of the mature protein and that this effect may be attributable, at least in part, to dephosphorylation of eIF2α.

**DISCUSSION**

We have shown that NFB42 and Skp1 interact with SHPS-1 both in mouse brain and in mouse melanocytes. NFB42 and Skp1 together with Cullin1 constitute the SCF<sup>NFB42</sup> ubiquitin ligase complex, which we have now shown mediates the polyubiquitination of immature SHPS-1 and its subsequent degradation by the ERAD pathway, a process important for the elimination of misfolded proteins generated under conditions of ER stress. Forced expression of NFB42 resulted in an increase...
SCF^{NFB42} facilitates elimination of SHPS-1 aggregates by the ERAD pathway. Melan-a cells seeded on glass coverslips were transiently transfected both with 1 μg of pEGFP N2 containing human SHPS-1 cDNA and with 1 μg of pCl-neo encoding Myc-tagged wild-type NFB42 or NFB\DeltaPAF (or with the empty vector) as indicated. Twenty-four hours after transfection, the cells were incubated for an additional 18 h with 10 μM MG132 or vehicle and then fixed. The recombinant SHPS-1 and NFB42 proteins were detected by autofluorescence of GFP (A–D) or by immunostaining with mAb 9E10 to the Myc tag (E–H). Merged images are shown in I and J. Original magnification, ×630.

FIG. 4. Effects of overexpression of NFB42 on the binding of SHPS-1 to G. nivalis agglutinin (GNA) lectin. A, CHO-K1 cells were transiently transfected with both 2 μg of pTracerCMV encoding SHPS-1 and 2 μg of pcDNA3 encoding (or not) HA-tagged wild-type NFB42 or NFB\DeltaPAF, as indicated. Cell lysates were prepared 48 h after transfection and were incubated with G. nivalis agglutinin-conjugated agarose beads. The precipitated proteins as well as cell lysates were then subjected to SDS-PAGE under reducing (left panel) or nonreducing (right panel) conditions followed by immunoblot analysis with polyclonal antibodies to SHPS-1. Cell lysates were also probed with antibodies to NFB42 (left panel). B, CHO-K1 cells were transfected as in A, 36 h after which they were incubated for an additional 2 h with 2 μM thapsigargin or vehicle. Cell lysates were then prepared and subjected to precipitation with G. nivalis agglutinin-conjugated agarose beads. The resulting precipitates were subjected to immunoblot analysis under reducing conditions with polyclonal antibodies to SHPS-1 (upper panel), and cell lysates were probed directly with antibodies to NFB42 (lower panel).
in the amount of SHPS-1 at the cell surface, indicating that activation of SCF\(^{NFB42}\) promotes the biosynthesis of SHPS-1. During the course of this study, Yoshida et al. (36) showed that SCF\(^{NFB42}\) targets N-glycosylated proteins. These researchers identified integrin \(\beta_1\) as a potential target of NFB42 and also showed that expression of inactive NFB42 delayed elimination of typical ERAD substrates, although the biological significance of these observations was unclear. Our results now establish SHPS-1 as a physiological substrate of SCF\(^{NFB42}\); moreover, they uncover a previously unidentified posttranslational mechanism by which the elimination of misfolded protein mediated by this ubiquitin ligase relieves the ER stress response.

Fig. 6. Effects of overexpression of NFB42 on the expression of SHPS-1 at the cell surface. A, CHO-K1 cells were transiently transfected both with 2 \(\mu\)g of pTracerCMV encoding SHPS-1 and with 2 \(\mu\)g of pcDNA3 encoding (or not) wild-type NFB42 or NFB\(\Delta\,\Delta\)F, as indicated. Thirty-six hours after transfection, the cells were detached from the culture dish and incubated with a mAb to mouse SHPS-1 or with control IgG (not shown). Immune complexes were then detected with Alexa Fluor 488-conjugated goat antibodies to rat IgG and flow cytometry. The percentages of SHPS-1-positive cells that exhibited strong (M1) or weak (M2) immunoreactivity are indicated. Data are representative of those obtained in three independent experiments. B, Melan-a cells were transiently transfected both with 3 \(\mu\)g of pSRe encoding eIF2\(\alpha\), eIF2\(\alpha\) phosphorlated on Ser51, and to NFB42 (upper panel). The amount of Ser\(^{51}\)-phosphorylated eIF2\(\alpha\) (eIF2\(\alpha\)-P) was quantified by scanning densitometry with the NIH Image program, normalized by the total amount of eIF2\(\alpha\), and expressed as a percentage of the value for cells transfected with both empty vectors (lower panel). Data are representative of those obtained in three independent experiments.
and supports the production of mature SHPS-1.

Several lines of evidence indicate that SCF NFB42 mediates elimination of SHPS-1 by the ERAD pathway. First, blockade of this pathway by the proteasomal inhibitor MG132 induced SHPS-1 to form aggresome-like structures in the cytoplasm, thus identifying SHPS-1 as an ERAD substrate. Second, NFB42 selectively associated with and mediated the polyubiquitination of immature forms of SHPS-1, and these events were enhanced by the inhibition of proteasome activity. Thus, SCFNFB42 likely acts as a bona fide E3 that targets immature SHPS-1 for proteasomal degradation. Third, expression of wild-type NFB42 suppressed, whereas that of the dominant negative mutant NFB/H9004F enhanced, the formation of cellular aggregates containing SHPS-1 in response to inhibition of the proteasome. Together, these results are consistent with the notion that NFB42 binds to misfolded forms of SHPS-1 generated during protein maturation, and they suggest a role for SCFNFB42 in elimination of the misfolded protein by the ERAD pathway.

NFB42 and Skp1 are both cytosolic proteins and thus would be expected to be separated topologically from the region of SHPS-1 where they associate. NFB42 recognizes the N-linked high mannose oligosaccharides of proteins as they are translocated from the ER to the cytosol (36). SHPS-1 is differentially glycosylated in a tissue-specific manner; neuronal (brain-derived) SHPS-1, unlike the myeloid (spleen-derived) type, is almost completely devoid of galactose in its N-glycan structures, although both types of SHPS-1 possess a high content of oligomannosidic moieties (37). It therefore seems likely that the mannosylated extracellular region of SHPS-1, when dislocated from the ER membrane, directly binds NFB42.

Elimination of misfolded SHPS-1 by SCFNFB42 resulted in a marked increase in the expression of the mature protein at the cell surface. If the rate of protein synthesis exceeds the capacity of the ER to process the newly synthesized protein molecules, protein folding becomes compromised, and the deployment of incompletely folded or assembled proteins may result in detrimental effects. Eukaryotic cells thus coordinate protein folding in the ER with gene transcription and mRNA translation by a process known as the unfolded protein response (UPR) (38–40). Furthermore, genetic evidence has revealed an essential regulatory link between ERAD and the UPR; dysfunction of the ubiquitin-proteasome system stabilizes ERAD substrates and results in persistent activation of the UPR (41, 42). On the basis of these previous observations and our present data, we propose a role for the SCFNFB42 ubiquitin ligase in the regulation of SHPS-1 biosynthesis (Fig. 7). Functional activation of SCFNFB42 thus likely facilitates elimination of misfolded SHPS-1 that accumulates during cellular stress and thereby restores the protein folding capacity of the ER, resulting in attenuation of the UPR, de-repression of mRNA translation, and increased surface expression of SHPS-1. This hypothesis is supported by our observation that forced expression of NFB42 prevented the formation of SHPS-1 aggregates in response to thapsigargin-induced ER stress. Furthermore, NFB42 expression induced the dephosphorylation of eIF2α, which is indicative of attenuation of the UPR, suggesting that eIF2α participates in the up-regulation of SHPS-1 expression. Given that incorrectly folded proteins might interfere with the transport of other normal proteins out of the ER (43), it is also possible that activation of SCFNFB42 facilitates SHPS-1 transport to the cell surface (Fig. 7).

**Fig. 7. Proposed role for the SCFNFB42 ubiquitin ligase in regulation of SHPS-1 biosynthesis.** Misfolded forms of SHPS-1 generated constitutively or in response to ER stress are either retained in the ER, where they might associate with molecular chaperone such as calnexin, or translocated to the cytosol. In the absence of NFB42 (right), they form aggresomes in the cytosol and induce the phosphorylation of eIF2α through the action of an ER-resident stress-responsive protein kinase (or kinases), resulting in cytotoxicity and inhibition of SHPS-1 mRNA translation, respectively. The misfolded proteins might also inhibit SHPS-1 trafficking along the secretory pathway. In the presence of NFB42 (left), however, these effects are likely counteracted by SCFNFB42-mediated elimination of the misfolded proteins by the ubiquitin-proteasome pathway. See text for further details. Note that intact SHPS-1 forms a cis dimer (H. Ohnishi and T. Matozaki, unpublished data).
Regulation of SHPS-1 Biosynthesis by Ubiquitination

The ERAD pathway protects cells against the pathological effects of protein aggregates (44), a role that is especially important in nonreplicating cells such as terminally differentiated neurons. Misfolding of the abundant glycoproteins of neuronal cells would thus be expected to exert toxic effects if not kept under control by this pathway. Given that terminal mannose neurons. Misfolding of the abundant glycoproteins of neuronal effects of protein aggregates (44), a role that is especially impor-

tant in nonreplicating cells such as terminally differentiated neurons through the elimination of misfolded glycoproteins.

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REFERENCES

1. Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda, M., Takada, T., and Kasuga, M. (1996) Mol. Cell. Biol. 16, 6887–6899
2. Yamao, T., Matozaki, T., Amano, K., Matsuda, Y., Takahashi, N., Ochi, F., Fujioka, Y., and Kasuga, M. (1997) Biochem. Biophys. Res. Commun. 231, 61–67
3. Kharitonenko, A., Chen, Z., Sures, I., Wang, H., Schilling, J., and Ulrich, A. (1997) Nature 386, 181–186
4. Sano, S., Ohnishi, H., Omore, A., Hasegawa, J., and Kubota, M. (1997) FEBS Lett. 411, 327–334
5. Sagninario, C., Sterling, H., Beckers, C., Kobayashi, R., Solimena, M., Ullu, E., and Vignery, A. (1998) Mol. Cell. Biol. 18, 6213–6223
6. Coma, S., Weng, W., Olinsky, S., Ishwad, P., Mi, Z., Hempel, J., Watkins, S., Lagenaur, C. F., and Narayanan, V. (1997) J. Neurosci. 17, 8702–8710
7. Jiang, P., Lagenaur, C. F., and Narayanan, V. (1999) J. Biol. Chem. 274, 559–562
8. Oldenborg, P. A., Zheleznjak, A., Fang, Y. F., Lagenaur, C. F., Gresham, H. D., and Lindberg, F. P. (2000) Science 288, 2051–2054
9. Han, X., Sterling, H., Chen, Y., Sagninario, C., Brown, E. J., Frazier, W. A., Lindberg, F. P., and Vignery, A. (2000) J. Biol. Chem. 275, 37984–37992
10. Steffiert, M., Brossart, P., Cant, C., Colla, M., Colonna, M., Brugger, W., Kanz, L., Ulrich, A., and Bühring, H. J. (2001) Blood 97, 2741–2749
11. Liu, Y., Bühring, H. J., Zen, K., Burst, L., Schnell, F. J., Williams, I. R., and Parkos, C. A. (2002) J. Biol. Chem. 277, 10028–10036
12. Timms, J. F., Swanson, K. D., Marie-Cardine, A., Raab, M., Rudd, C. E., Schraven, B., and Neel, B. G. (1999) Curr. Biol. 9, 927–930
13. Cant, C. A., and Ulrich, A. (2001) Cell. Mol. Life Sci. 58, 117–124
14. Oshima, K., Ruohu Amin, A. R., Suzuki, A., Hamaguchi, M., and Matsuda, S. (2002) FEBS Lett. 519, 1–7
15. Inagaki, K., Yamao, T., Noguchi, T., Matozaki, T., Fukunaga, K., Takada, T., Hossokta, T., Akira, S., and Kasuga, M. (2000) EMBO J. 19, 6721–6731
16. Yamao, T., Noguchi, T., Takeuchi, O., Nishiyama, U., Morita, H., Hagiwara, T., Akahori, H., Kato, T., Inagaki, K., Okazawa, H., Hayashi, Y., Matozaki, T., Takeda, K., Akira, S., and Kasuga, M. (2002) J. Biol. Chem. 277, 39833–39839
17. Mi, Z. P., Jiang, P., Weng, W. L., Lindberg, F. P., Narayanan, V., and Lagenaur, C. F. (2000) J. Comp. Neurol. 416, 335–344
18. Machida, K., Matsuura, S., Yamaki, R., Senga, T., Thant, A. A., Kurata, H., Miyazaki, K., Hayashi, K., Okuda, T., Kitamura, T., Hayakawa, T., and Hamaguchi, M. (2000) Oncogene 19, 1710–1718
19. Erhardt, J. A., Hynacka, W., Dillenedetto, A., Shen, N., Stone, N., Paulson, H., and Pittman, R. N. (1998) J. Biol. Chem. 273, 35222–35227
20. Craig, K. L., and Tyers, M. (1999) Prog. Biophys. Mol. Biol. 72, 299–328
21. Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Eilledge, S. J. (1996) Cell 86, 263–274
22. Seel, J., Shevchenko, A., Shevchenko, A., and Deshaies, R. J. (2001) Nat. Cell Biol. 4, 384–391
23. Takeda, T., Matozaki, T., Takeda, H., Fukunaga, K., Noguchi, T., Fujioka, Y., Okazaki, I., Tsuda, M., Yamao, T., Ochi, F., and Kasuga, M. (1998) J. Biol. Chem. 273, 9234–9242
24. Chung, W., and Lagenaur, C. F. (1999) Dev. Biol. 137, 219–222
25. Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R. A., and Nakayama, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3859–3863
26. Bennett, D. C., Cooper, P. J., and Hart, J. R. (1987) Int. J. Cancer 39, 414–418
27. Deshaies, R. J. (1999) Annu. Rev. Cell Dev. Biol. 15, 435–467
28. Bonicafico, J. S., and Weissman, A. M. (1998) Annu. Rev. Cell Dev. Biol. 14, 19–57
29. Elgar, G., and Helenius, A. (2001) Curr. Opin. Cell Biol. 13, 431–437
30. Hampton, R. Y. (2002) Curr. Opin. Cell Biol. 14, 476–482
31. Johnston, A. J., Ward, C. L., and Kopito, R. R. (1998) J. Cell Biol. 143, 1883–1899
32. Kopito, R. R. (2000) Trends Cell Biol. 10, 524–530
33. Shibuya, N., Goldstein, I. J., Van Damme, J. E., and Peumans, W. J. (1988) J. Biol. Chem. 263, 728–734
34. Prevel, C. R., Brostrom, M. A., and Brostrom, C. O. (1993) Mol. Cell. Biochem. 128, 255–265
35. Wang, W. L., Brostrom, M. A., Kuznetsov, G., Gnutzer-Yellen, D., and Brostrom, C. O. (1993) Biochem. J. 299, 71–79
36. Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (2002) Nature 418, 142–144
37. van den Nieuwenhui, J. M., Renardel de Lavalette, C., Diaz, N., van Die, I., and van den Berg, T. K. (2001) J. Cell Sci. 114, 1321–1329
38. Kaufman, R. J. (1999) Genes Dev. 13, 1211–1233
39. Mori, K. (2000) Cell 101, 451–454
40. Patil, C., and Walter, P. (2001) Curr. Opin. Cell Biol. 13, 349–355
41. Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000) Nat. Cell Biol. 2, 379–384
42. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000) Cell 101, 249–258
43. Graves, T. K., Patel, S., Dannies, P. S., and Hinkle, P. M. (2001) J. Cell Sci. 114, 3685–3694
44. Bence, N. F., Sampa, R. M., and Kopito, R. R. (2001) Science 292, 1552–1555

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