Inositol 1,4,5-trisphosphate (IP₃) receptors are endoplasmic reticulum (ER) membrane calcium channels that, upon activation, become substrates for the ER-associated degradation (ERAD) pathway. Although it is clear that IP₃ receptors are polyubiquitinated upon activation and are transferred to the proteasome by a p97-based complex, currently nothing is known about the proteins that initially select activated IP₃ receptors for ERAD. Here, we sought to identify novel proteins that associate with and mediate the ERAD of endogenous activated IP₃ receptors. SPFH2, an uncharacterized SPFH domain-containing protein, rapidly associated with IP₃ receptors in a manner that preceded significant polyubiquitination and the processing of other ERAD substrates. Overall, these studies identify SPFH2 as a key ERAD pathway component and suggest that it may act as a substrate recognition factor.

The endoplasmic reticulum (ER)²-associated degradation (ERAD) pathway is responsible for the degradation of aberrant proteins in the ER (1) and, in addition to this “quality control” function, also accounts for the degradation of several metabolically regulated, native ER proteins (2, 3). The essential features of the ERAD pathway are substrate recognition, polyubiquitination, and delivery to the 26 S proteasome, which is located in the cytosol (1). Much of our understanding of the ERAD pathway has been obtained using yeast as a model system, and although there are many parallels between the yeast and mammalian ERAD pathways, there appear to be some key differences. Most notably, mammalian cells have additional components that add diversity and complexity to substrate recognition and processing (4).

Quite a lot is known about how ERAD substrates are polyubiquitinated and transferred to the proteasome. Studies in yeast suggest that ER luminal substrates and membrane substrates with aberrant luminal or membrane domains are polyubiquitinated by an ER membrane protein complex containing the ubiquitin ligase (E3) Hrd1p, whereas membrane substrates with aberrant cytosolic domains are targeted by a complex containing the E3 Doa10p (4). Hrd3p binds to and regulates Hrd1p (5), and together with the ER luminal lectin Yos9p, may use its large luminal domain to recruit ERAD substrates to a putative “retrotranslocation” channel in the ER membrane (6 – 8). Retrotranslocation is facilitated by the cytosolic Cdc48p-Ufd1p-Npl4p complex, which associates with Hrd1p and Doa10p via membrane-bound Ubx2p (4, 8 – 10), and likely uses ATP hydrolysis to both unfold ERAD substrates and extract them from the ER membrane (11 – 13). Polyubiquitination then occurs either simultaneously with or immediately after retrotranslocation (14, 15).

Further, the mammalian homologs of many of these proteins have been identified and appear to be organized similarly (4). The mammalian E3s Hrd1p and gp78 are homologous to yeast Hrd1p, and each polyubiquitines several ERAD substrates (16 – 18). The mammalian homolog of Hrd3p, SEL1L, complexes with Hrd1p and may also contribute to ERAD substrate processing (19, 20). The mammalian p97-Ufd1p-Npl4p complex has properties similar to its yeast counterpart (11, 21, 22), and the mammalian homolog of Ubx2p helps anchor it to the ER membrane (23). Finally, Derlin-1, -2, and -3, homologs of yeast Der1p, have been suggested to form the retrotranslocation channel in the ER membrane (19, 24, 25).

In contrast, how proteins are initially recognized for ERAD is poorly understood, and emerging evidence points to the existence of multiple recognition factors and mechanisms rather than a single unified scheme (26). For example, in yeast, Yos9p, together with Kar2p and Hrd3p, selectively binds to and targets a range of terminally misfolded luminal glycoproteins to the Hrd1p complex (4, 6, 7). Likewise, the mammalian homologs of Kar2p (11) and Hrd3p (20), as well as the mammalian EDEM

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**References:**

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2. The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; siRNA, short interfering RNA; E3, ubiquitin-protein isopeptide ligase; HMG, hydroxy-3-methylglutaryl-coenzyme A reductase; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; GnRH, gonadotropin-releasing hormone; ET1, endothelin 1; RNAi, RNA interference; HA, hemagglutinin; TNF, tumor necrosis factor; Endo H, endoglycosidase H; DTT, dithiothreitol; PBS, phosphate-buffered saline; DRM, detergent-resistant membrane.

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**Figure:**

The figure shows a schematic representation of the mammalian ERAD pathway, highlighting the roles of various proteins and complexes in the degradation process. The figure includes a cartoon of the ER membrane, with proteins and complexes depicted in different colors to indicate their functions and interactions. The figure explains the sequential steps of substrate recognition, polyubiquitination, and retrotranslocation, emphasizing the critical role of SPFH2 in this process.
proteins (27), appear to interact directly with glycoproteins destined for ERAD. Alternatively, substrate recognition can be mediated by factors that select specific or highly restricted groups of proteins for degradation. For example, the mammalian INSIGs bind directly to 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), and this interaction recruits factors, including the E3 gp78, that are required for the cholesterol-induced ERAD of HMGR (18, 28). Given the diversity of proteins that are targeted for ERAD, it is likely that additional substrate recognition factors will emerge.

Inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) form tetrameric, IP₃- and Ca²⁺-gated Ca²⁺ channels in ER membranes and play a key role in cell signaling (29, 30). Stimulation of certain cell surface receptors triggers IP₃ formation at the plasma membrane, which then diffuses through the cytosol and binds to IP₃Rs (29). This, in concert with Ca²⁺ binding, induces conformational changes in the tetrameric channel that permit Ca²⁺ to flow from stores within the ER lumen into the cytosol (31, 32). There are three IP₃R types in mammals, IP₃R1, IP₃R2, and IP₃R3, and although they differ considerably in their tissue distribution, they have similar properties, are often co-expressed, and can form homo- or heterotetramers (30, 33). Remarkably, activation of endogenous IP₃Rs leads to their rapid polyubiquitination and subsequent degradation by the 26 S proteasome (34–36), a phenomenon that has been demonstrated in many mammalian cell types, including cholecystokinin-stimulated pancreatic acinar cells (37), gonadotropin-releasing hormone (GnRH)-stimulated αT3-1 mouse pituitary gonadotropes (38), and endothelin 1 (ET1)-stimulated Rat-1 primary fibroblasts (39). The ERAD pathway seems to be responsible for this process, because the ubiquitin-conjugating enzyme that ubiquitinitates IP₃Rs is mamUbc7 (3), an enzyme widely implicated in ERAD (2, 40), and the p97-Ufd1-Npl4 complex mediates the degradation of polyubiquitinated IP₃Rs (39). Importantly, endogenous IP₃Rs represent a unique tool for studying ERAD, because activation almost instantaneously converts them from their native form into ERAD substrates (36, 39). This contrasts with the substrates typically used to study ERAD in mammalian cells, for example, the T-cell receptor subunits TCRα and CD3δ, which are constitutively degraded and usually overexpressed by transfection (40).

Here, we studied endogenous IP₃Rs in stimulated mammalian cells in an effort to identify novel proteins that might be involved in the ERAD of IP₃Rs and perhaps other substrates. We discovered that an uncharacterized protein, known as SPFH domain-containing protein, member 2 (SPFH2), associates very rapidly with activated IP₃Rs in a variety of cell types, that a proportion of cellular SPFH2 is associated with several established ERAD pathway components, and that RNA interference (RNAi)-mediated depletion of SPFH2 inhibits IP₃R1 polyubiquitination and degradation and the turnover of model ERAD substrates. SPFH2 shares homology with a diverse family of proteins that contain an SPFH domain, an ~200 amino acid motif of unknown function, named for the original family members: Stomatin, Prohibitin, Flotillin, and HFlc/HflK (41–43). Overall, these studies identify SPFH2 as a key ERAD pathway component in mammalian cells and suggest that it may act as a substrate recognition factor.

**EXPERIMENTAL PROCEDURES**

**Materials**—αT3-1 and Rat-1 cells were routinely cultured as described (39), and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Already-available antibodies used were: rabbit polyclonal anti-IP₃R1, anti-IP₃R2, and anti-IP₃R3 (33), anti-α-transaldolase (a kind gift from Dr. Andras Perl, State University of New York Upstate Medical University), anti-Derlin-1 and anti-sec61β (kind gifts from Dr. T. Rapoport, Harvard Medical School, Boston, MA), rat monoclonal anti-grp94 (StressGen), mouse monoclonal anti-mono- and -polyubiquitinated conjugates, clone FK2 (Bio-Mol International), anti-β-actin (Sigma), anti-p97 (Research Diagnostics, Inc.), anti-Ufd1, anti-Ufd2, anti-hsp90, anti-IP₃R3, and anti-αko (BD Transduction Laboratories), anti-hemagglutinin (HA) epitope, clone HA11 (Covance), and horseradish peroxidase- and fluorophore-conjugated secondary antibodies (Sigma). Rabbit polyclonal anti-gp78, anti-Hrd1, and anti-SPFH2 were generated against peptides corresponding to the C terminus of each protein (DPVTLLRRMILAAFERLQ, RLQKLESPVAH, and DKLGFDLEPLEATAKDN, respectively) and were affinity-purified as described (33). SDS, Triton X-100, protease inhibitors, N-ethylmaleimide, GnrH, cholecystokinin, digitonin, Polybrene (hexamethrine bromide), puromycin, cycloheximide, and TnFα were purchased from Sigma; ET1 was from Calbiochem; T4 DNA ligase, calf intestinal alkaline phosphatase, all restriction enzymes, and endoglycosidase H (Endo H) were from New England Biolabs; Precision Plus™ Protein Standards, DTT, and bisacrylamide were from Bio-Rad; G418 was from Gibco-BRL; doxycycline was from Clontech; Protein A-Sepharose CL-4B was from Amersham Biosciences; Lipopectamine 2000 was from Invitrogen; and tetraborom (PS-341) was a gift from Millennium Pharmaceuticals.

**Cell Lysis and Immunoprecipitation**—For αT3-1 cells, near-confluent monolayers in 15-cm dishes were incubated without or with GnRH, and cells were harvested by adding lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8.0) containing a protease inhibitor mixture (0.2 mM phenylmethylsulfonfluoride, 10 μM leupeptin, 10 μM pepstatin, 0.2 μM soybean trypsin inhibitor) and 1 mM DTT, pipetting the cells off the dish, and incubating at 4 °C for 30 min. Lysates were clarified by centrifugation (16,000 × g for 10 min at 4 °C), and either samples were removed and mixed with gel loading buffer (35) for subsequent immunoblotting or selected proteins were immunoprecipitated by incubating with antisera and Protein A-Sepharose CL-4B for 4–16 h at 4 °C. Immunoprecipitates were washed thoroughly with lysis buffer, resuspended in gel loading buffer, subjected to SDS-PAGE, and either transferred to nitrocellulose for immunoblotting or silver stained with the Proteosilver™ Plus Silver Stain Kit (Sigma). For Rat-1 cells, near-confluent monolayers in 15-cm dishes or 6-well plates were serum-starved for 16 h, and then incubated with or without ET1. Lysates were harvested by adding lysis buffer plus protease inhibitor mixture, followed by vigorous scraping. Cells were then treated with 2.5 mM N-ethylmaleimide for 1 min to inhibit de-ubiquitinating enzymes, followed by 5 mM DTT.
After 30 min at 4 °C, lysates were clarified by centrifugation, and IP₃-R1 was either analyzed in immunobLOTS or immunoprecipitated and analyzed as for αT₃-1 cells. For pancreas, pancreatic tissue was removed from female rats and cross-chopped into 0.5-mm³ cubes with a McIlwain chopper, and the cubes were washed several times with Oₐ-gassed 10 mM HEPES (pH 7.4), 127 mM NaCl, 0.57 mM MgCl₂, 4.7 mM KCl, 0.55 mM Na₂PO₄, 6 mM NaOH, 1.3 mM CaCl₂, 11.1 mM glucose, 0.05 mg/ml soybean trypsin inhibitor, and 5 mg/ml bovine serum albumin. Samples were then incubated at 37 °C in the same buffer without or with cholecystokinin. The tubes were then homogenized in lysis buffer plus protease inhibitor mixture as described (37) and incubated with a mixture of anti-IP₃-R1, anti-IP₃-R2, and anti-IP₃-R3 to immunoprecipitate all receptor types, which were then analyzed as for αT₃-1 cells. For TNFα experiments in HeLa cells, near-confluent monolayers were incubated without or with TNFα, and cells were harvested by adding lysis buffer plus protease inhibitor mixture and pipetting the cells off the dish. Lysates were then treated with 2.5 mM N-ethylmaleimide for 1 min, followed by 5 mM DTT. After 30 min at 4 °C, lysates were clarified by centrifugation and either samples were taken for immunoblotting or 1kBα was immunoprecipitated as described for αT₃-1 cells.

**Electrophoresis, Immunoblotting and Mass Spectroscopy**—Samples in gel loading buffer (35) were routinely heated to 100 °C for 3 min prior to loading onto gels, but when HMG R₃₅₀-3HA and CD38-HA were analyzed, samples were heated at 37 °C for 30 min to avoid protein aggregation. After SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunoblotted, and immunoreactivity was detected with Pierce chemiluminescence reagents (Fisher Scientific) and quantitated using a GeneGenome Imager (Syngene Bio Imaging). Alternatively, silver-stained protein bands were cut from SDS-PAGE gels, destained, and sent for in-gel trypsinization and mass spectral analysis at the Molecular Biology Core Facilities at Dana Farber Cancer Institute (Boston, MA). The MS-Fit database (University of California San Francisco Mass Spectrometry Facility) was used to provide possible identities for the peptides generated from each gel band. Resulting identities were confirmed by immunoblotting.

**Plasmids and siRNA Constructs**—An expressed sequence tag clone containing the full-length mouse SPFH2 sequence in pCMV-Sport6 was purchased from ATCC and verified by sequencing, and the open reading frame was cloned into pcDNA3 (Invitrogen). SPFH2 was tagged with an HA epitope at its C terminus by using PCR to insert the HA epitope sequence in-frame with the 3’-end of SPFH2 cDNA, and SPFH2-HA-N106Q was generated using the Stratagene QuikChange site-directed mutagenesis kit. HA ubiquitin cDNA was a kind gift from Dr. R. Rottapel (Ontario Cancer Institute, Toronto, Canada), CD38-HA cDNA was a kind gift from Dr. A. Weissman (NCI, National Institutes of Health, Frederick, MD), and HMGR₉₀-3HA cDNA was a kind gift from Dr. J. Roitman (Sheba Medical Center, Tel Hashomer, Israel). Five short interfering RNA (siRNA) sequences (SPFH2si1–5) were designed to correspond to different regions of rat, mouse, and human SPFH2 mRNA, and two of these, SPFH2si1, encoded by tgtgaaggg-gtgaaccttcct, and SPFH2si5, encoded by ctgcaacctgataagta, were found to be effective. In addition, a control siRNA (Random), encoded by actgtcagaatctacca, was designed to have no homology to any rat, mouse, or human mRNA sequences. Sense (5’gatccccc(SPFH2si1–5)ttaagaag(reverse complement)tttttg-gaa) and antisense (5’agctttccccaa(SPFH2si1–5)ttcttggagag(reverse complement)ggg) oligonucleotides were synthesized (Sigma-Genosys), annealed, and ligated into the retroviral vectors, pSUPER.retro.puro (44, 45), or, for doxycycline-inducible expression, 6OH₁-O-pSUPER.retro.puro. Correct ligation of these siRNA-encoding vectors was confirmed by restriction digestion and DNA sequencing. 6OH₁-O-pSUPER.retro.puro was generated by replacing the H₁ promoter and siRNA-encoding region of pSUPER.retro.puro with the H₁ promoter-tetracycline operator (T₄⁺)-siRNA-encoding sequence region of pTER (a kind gift from Dr. H. Clevers, Center for Biomedical Genetics, Utrecht, The Netherlands) (46). To further decrease transcription in the absence of doxycycline, six T₅₈ were introduced upstream of the H₁ promoter. Upon introduction into cells, transcription from the H₁ RNA polymerase III promoter in each of these vectors generates short hairpin RNAs that are further processed into siRNAs by the cell’s endogenous RNAi machinery (45).

**Immunofluorescence Microscopy**—HeLa cells plated on poly-L-lysine-coated coverslips were transiently co-transfected with pDsRed2-ER (Clontech) and SPFH2 cDNAs using Lipofectamine 2000. 24 h post-transfection, the cells were fixed in 1% paraformaldehyde by 1 h, washed in PBS for 5 min, incubated in −20 °C methanol for 10 min, rinsed in −20 °C acetone, washed in PBS for 5 min, incubated in goat blocking solution (10% goat serum, 0.1% bovine serum albumin in PBS) for 45 min at room temperature, incubated with anti-SPFH2 (1:200 in goat blocking solution) for 16 h at 4 °C, washed thrice in PBS for 5 min, and then incubated with fluorescein isothiocyanate anti-rabbit (1:200 in goat blocking solution) for 1 h. After three-5-min washes in PBS, the cells were mounted on glass slides using Vectashield mounting medium (Vector Laboratories) to preserve the fluorescent signals. Images were acquired on a Zeiss AxioPlan 2 microscope equipped with a 63× oil immersion objective (Appochromat from Zeiss).

**Cell Fractionation and Proteinase K Incubation**—For cell fractionation, Rat-1 cells were harvested in 1.5 ml of hypotonic homogenization buffer (10 mM Tris-base, 1 mM EGTA, pH 7.4) containing protease inhibitor mixture and 1 mM DTT, and sonicated for 1 min. The homogenates were first centrifuged at 20,000 × g for 1 h at 4 °C, and the pellets were resuspended in 1.5 ml of either hypotonic buffer or hypotonic buffer containing 1% Triton X-100, 0.5% SDS, or 0.1 M Na₂CO₃, pH 11.2, incubated for 1 h at 4 °C with frequent vortexing, and then re-centrifuged at 100,000 × g for 1 h at 4 °C to obtain soluble and pellet fractions. Pellets were resuspended in 1.5 ml of the appropriate buffer, and equal volumes of each fraction were loaded onto gels. α-Transaldolase was used as a cytosolic marker (3). For the proteinase K experiments, HeLa cells were trypsinized and resuspended in ice-cold PBS containing either no detergent, 100 μg/ml digitonin, or 0.5% Triton X-100 and incubated for 10 min at 4 °C. Proteinase K (Invitrogen) was added at the indicated concentration, and the samples were incubated for an additional 30 min at 4 °C. Reactions were quenched with 1 mM phenylmethylsulfonyl fluoride.
Stable RNAi and SPFH2 Re-introduction in Rat-1 Cells—Retroviruses were generated by transfecting 293T cells using calcium phosphate with each 6OH1O-pSUPER.retro.puro plasmid together with pVPack-Eco and pVPack-GP, plasmids that encode for Moloney murine leukemia viral envelope and Gag-Pol proteins (Stratagene). 48 h post-transfection, the culture media was passed through 0.45-μm filters (Nalgene), and the virus-containing filtrate was aliquoted, quick-frozen on dry ice, and stored at −80 °C. Viral titers were determined by transducing Rat-1 cells with serial dilutions of each viral stock and counting colonies that survived in 2.5 μg/ml puromycin. Rat-1 cells stably expressing tTS, a fusion protein of the Tet repressor and the KRAB-AB transcriptional silencing domain, were generated by incubating cells with retrovirus containing the pQC-tTS-IN vector (Clontech), selecting in 300 μg/ml G418, and isolating individual clones. One clone was then expanded and seeded at 4 × 10⁴/well in a 12-well plate, and 24 h later, was incubated with equal titers of each 6OH1O-pSUPER.retro.puro-containing retrovirus (>10⁵ colony forming units/ml) supplemented with 8 μg/ml Polybrene for ~8 h, followed by incubation in fresh medium for 16 h. To induce expression of the siRNAs, transduced cells were replated and treated with 1 μg/ml doxycycline for 48 h, followed by selection in 2.5 μg/ml puromycin for at least 24 h. Cells were then processed to determine the levels of endogenous SPFH2 and other proteins, or IP₃R1 polyubiquitination and down-regulation. Cytosolic Ca²⁺ concentration was measured by loading the cells with 10 μM Fura-2-AM as described (39). Re-introduction of SPFH2 was accomplished using a mouse SPFH2 CDNA construct (SPFH2–5*) containing five silent mutations that render the transcribed mRNA refractory to SPFH2si5, generated using the Stratagene QuikChange site-directed mutagenesis kit. Cells expressing Random siRNA or SPFH2si5 were transiently co-transfected with vectors encoding HA-ubiquitin and either pcDNA3 or control siRNA or SPFH2si5 were transiently co-transfected using Lipofectamine 2000 with pSUPER.retro.puro vectors encoding either Random (1.6 μg) or SPFH2si5 (0.8 μg of each) siRNAs. Preliminary studies showed that this combination of SPFH2si1 and SPFH2si5 produced optimal SPFH2 depletion. After 24 h, the medium was replaced with medium supplemented with puromycin (1 μg/ml), and 24 h later, cells were transiently transfected again with 1 μg of HMG₃₅₀–3HA or CD₃₈–HA in puromycin-free medium. After 24 h, cells were incubated with 20 μg/ml cycloheximide for various times and then harvested by incubation in 155 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4, and centrifugation (16,000 × g for 1 min at room temperature). Pellets were then solubilized in lysis buffer plus protease inhibitor mixture and 1 mM DTT, incubated for 30 min at 4 °C, centrifuged (16,000 × g for 10 min at 4 °C), and samples were probed in immunoblots with anti-HA epitope to detect HMG₃₅₀–3HA and CD₃₈–HA.

FIGURE 1. SPFH2 is a novel IP₃R interacting protein. A, αT3-1 cells were treated with 100 nM GnRH for the indicated times. Cell lysates were incubated with anti-IP₃R1, and the immunoprecipitates were probed in immunoblots for ubiquitin, IP₃R1, p97, gp78, Ufd1, and SPFH2. Quantitated data for IP₃R1 polyubiquitination and SPFH2 and p97 co-immunoprecipitation are graphed (n = 3; *, denotes p < 0.05 comparing p97 and SPFH2 immunoreactivity); p97, gp78, and Ufd1 all co-immunoprecipitated with IP₃R1 with similar dynamics, so only the data for p97 are shown. B, rat pancreatic tissue was treated without (−) or with (+) 200 nM cholecystokinin for 10 min, and IP₃Rs were immunoprecipitated and probed in immunoblots for ubiquitin, IP₃R3, and SPFH2.

Data Presentation and Analysis—All experiments were repeated at least once, and representative images of gels or micrographs are shown. Quantitated data are graphed as mean ± S.E. of n independent experiments, with unpaired Student’s t test used to obtain p values.

RESULTS

SPFH2 Associates with Activated IP₃Rs—The known capacity of proteins that mediate ERAD (e.g. the p97-Ufd1-Npl4 complex) to associate with activated IP₃Rs (39) motivated us to...
examine whether novel mediators might also associate. Thus, we incubated αT3-1 cells with or without GnRH for 10 min, which induces robust IP$_{3}$R1 polyubiquitination and down-regulation (38), and silver stained anti-IP$_{3}$R1 immunoprecipitates. Two proteins that co-immunoprecipitated with IP$_{3}$R1 specifically in stimulated cells were readily stained and clearly predominant: one at 97 kDa and one at 43 kDa (data not shown). We analyzed these protein bands by mass spectrometry and defined the 97-kDa band as p97, and the 43-kDa band as an uncharacterized protein, known as SPFH2. To study the association dynamics of these proteins with activated IP$_{3}$R1, we stimulated αT3-1 cells with GnRH for various times, immunoprecipitated IP$_{3}$R1, and probed for p97 and SPFH2 and other, less abundant proteins shown previously in immunoblots to interact with activated IP$_{3}$R1 (Fig. 1A) (39). p97, gp78, and Ufd1 all co-immunoprecipitated with IP$_{3}$R1 in a manner that paralleled IP$_{3}$R1 polyubiquitination, peaking at 5–7 min, and consistent with the fact that all of these proteins contain ubiquitin-binding domains (16, 21, 22). In contrast, the peak interaction of SPFH2 with IP$_{3}$R1 occurred prior to peak polyubiquitination, and SPFH2 was strongly associated with IP$_{3}$R1 as little as 30 s after GnRH addition. These data indicate that SPFH2 binds to IP$_{3}$R1 as soon as it has been activated and independently of the degree of its polyubiquitination. To examine whether the interaction between SPFH2 and IP$_{R}$s is physiological, we treated rat pancreatic tissue with cholecystokinin, a secretagogue that stimulates IP$_{3}$R polyubiquitination and down-regulation (37), and again found that SPFH2 co-immunoprecipitated with activated IP$_{3}$Rs (Fig. 1B).

Characterization of SPFH2—SPFH2 belongs to a diverse class of proteins containing an SPFH domain, also known as the Prohibitin or Band 7 domain (41–43). The SPFH domain is loosely defined, and in fact, SPFH2 shares only limited homology with the other mammalian proteins in this class; human SPFH2 is 40% similar to the human flotillin (reggie) proteins, 25% similar to the human prohibitin proteins, and 15% similar to the human stomatin and stomatin-like proteins (full-length sequences analyzed by ClustalW alignment, data not shown). The function of the SPFH domain is currently unknown, and although the proteins that belong to this class are widely distributed throughout the cell, they do share some common features; notably, localization to membranes and...
detergent-insoluble lipid rafts via N-terminal hydrophobic regions, formation of oligomers, and, often, involvement in the regulation of membrane-associated proteases (47–54). SPFH2 is well conserved across species; the human, mouse, and rat homologs are 97% identical, and human SPFH2 shares ~55% identity and ~80% similarity with a similar protein from *Arabidopsis thaliana*, its most distant relative according to searches performed within the NCBI data base (Fig. 2A). Interestingly, however, there are no yeast homologs of SPFH2. Analyses performed within the NCBI data base (Fig. 2A, lanes 1 and 2), in several buffers to separate peripheral and integral membrane proteins by further centrifugation (Fig. 2B, lanes 3–10). Peripheral membrane proteins, for example, p97 (11), were partially removed from the pellet by further incubation in hypotonic buffer or Na2CO3, whereas integral membrane proteins were only solubilized by detergents; the polytopic ER membrane proteins, Hrd1 and gp78, were almost completely solubilized by 1% Triton X-100 and entirely solubilized by 0.1% SDS. Likewise, SPFH2 was removed from the pellet only by detergents, indicating that it is indeed an integral membrane protein.

We next examined the membrane topology of SPFH2. SPFH2 was not degraded or reduced in size by proteinase K after permeabilization of the plasma membrane with digitonin (14, 55) but was degraded after treatment with Triton X-100 (Fig. 3C). Thus, essentially all of SPFH2, including its C terminus, is located in the ER lumen. Probing with antisera directed against the C terminus of Hrd1, which is located on the cytoplasmic face of the ER membrane, confirmed that digitonin was effective in permeabilizing the plasma membrane. In contrast, the ER luminal protein grp94 was stable in digitonin-permeabilized cells but was degraded after treatment with Triton X-100, confirming that only Triton X-100 permeabilized the ER membrane. Thus, SPFH2 is a type II ER membrane protein, with a short N-terminal segment (~3 amino acids) exposed to the cytosol (Fig. 2A) and its C terminus, along with the bulk of the protein, located within the ER lumen.

**FIGURE 3.** SPFH2 is an ER membrane protein largely located within the ER lumen. A, HeLa cells co-transfected with either pcDNA3 (empty vector) plus the ER marker pDsRed2-ER (panels a and d), or cDNAs encoding SPFH2 plus pDsRed2-ER (panels c and d) were fixed in 1% paraformaldehyde and then permeabilized with methanol/acetonitrile. The cells were then labeled with anti-SPFH2 followed by fluorescein isothiocyanate anti-rabbit. Exposure times were either 600 ms (panel a) or 100 ms (panels b–d). B, Rat-1 fibroblasts were harvested in hypotonic buffer, sonicated, and fractionated by centrifugation at 20,000 × g (lanes 1 and 2). The resulting pellets were resuspended in either hypotonic buffer, 1% Triton X-100, 0.1% SDS, or 0.1 M Na2CO3, pH 11.2, as indicated, and re-centrifuged at 100,000 × g (lanes 3–10). The supernatants (S) and pellets (P) from each of these centrifugations were then probed for the indicated proteins. C, HeLa cells were incubated with the indicated detergents for 10 min at 4 °C, and then proteinase K was added at the indicated concentrations for 30 min at 4 °C. Samples were probed for grp94, Hrd1, and SPFH2. D, HeLa cells were transfected with either pcDNA3 (lanes 1 and 2) or cDNAs encoding SPFH2-HA (lanes 3 and 4) or SPFH2-HA-N106Q (lanes 5 and 6), and cell lysates were incubated without (−) or with (+) Endo H for 3 h and then probed for SPFH2 (lanes 1 and 2) or for HA epitope (lanes 3–6). E, SPFH2 is depicted, with the ER luminal SPFH domain indicated by the gray box, the N-linked glycosylation site at Asn-106 indicated by the asterisk, and the C-terminal epitope that is recognized by anti-SPFH2 indicated by the black box.

**SPFH2 Involvement in the Mammalian ERAD Pathway**

SPFH2 was targeted to the ER by an N-terminal calreticulin ER targeting sequence and a C-terminal KDEL ER retention signal (Fig. 3A, panel b). The same ER-like distribution was also seen in HeLa cells expressing exogenous SPFH2 (Fig. 3A, panels c and d), and for endogenous SPFH2 in αT3-1 and Rat-1 cells (data not shown), cells that were used in subsequent experiments. Interestingly, the ER-like distribution of SPFH2 was only evident if fixed cells were permeabilized with methanol/acetonitrile (Fig. 3A) or Triton X-100 but not with digitonin (data not shown). Because digitonin selectively permeabilizes the plasma membrane but not intracellular membranes, this suggests that the SPFH2 C-terminal epitope is located within the ER lumen.

To examine whether SPFH2 is an integral membrane protein, we fractionated Rat-1 cells by hypotonic lysis and centrifugation and resuspended the pellet, which contained SPFH2 but not the cytosolic marker, α-transaldolase (Fig. 3B, lanes 1 and 2), in several buffers to separate peripheral and integral membrane proteins by further centrifugation (Fig. 3B, lanes 3–10). Peripheral membrane proteins, for example, p97 (11), were partially removed from the pellet by further incubation in hypotonic buffer or Na2CO3, whereas integral membrane proteins were only solubilized by detergents; the polytopic ER membrane proteins, Hrd1 and gp78, were almost completely solubilized by 1% Triton X-100 and entirely solubilized by 0.1% SDS. Likewise, SPFH2 was removed from the pellet only by detergents, indicating that it is indeed an integral membrane protein.

To define the subcellular distribution of SPFH2, we employed HeLa cells, because they can be readily transfected. Staining with anti-SPFH2, which is directed against the C-terminal 19 amino acids of the protein, revealed an ER-like distribution for endogenous SPFH2 (Fig. 3A, panel a), identical to that of the ER marker, DsRed2-ER, a red fluorescent protein targeted to the ER by an N-terminal calreticulin ER targeting...
SPFH2 Involvement in the Mammalian ERAD Pathway

Because SPFH2 contains an N-linked glycosylation consensus sequence at asparagine 106 (Fig. 2A), we examined whether it is a glycoprotein. Treatment of HeLa cell lysates with Endo H led to a decrease of ~2 kDa in the apparent molecular mass of endogenous SPFH2, consistent with the loss of a single glycan (Fig. 3D, lanes 1 and 2). Next, we expressed in HeLa cells constructs encoding wild-type SPFH2 (SPFH2-HA) and SPFH2 with Asn-106 mutated to glutamine (SPFH2-HA-N106Q). SPFH2-HA ran at ~45 kDa (Fig. 3D, lane 3), and Endo H again caused an ~2-kDa downward shift, consistent with the loss of a single glycan (Fig. 3D, lane 4). In contrast, SPFH2-HA-N106Q migrated at ~43 kDa and was unaffected by Endo H (Fig. 3D, lanes 5 and 6). Thus, SPFH2 is modified by a single N-linked glycan at Asn-106, and this is consistent with the majority of SPFH2 being located within the ER lumen. A diagram summarizing the basic molecular architecture of SPFH2 is shown in Fig. 3E.

SPFH2 Is Associated with Components of the ERAD Pathway—Because SPFH2 binds to activated IP₃Rs, which are subsequently processed by the ERAD pathway, we examined whether polyubiquitin confirmed the presence of polyubiquitinated IP₃R1 (Fig. 4A, lanes 2 and 3). This verifies the results shown in Fig. 1A, where SPFH2 co-immunoprecipitated with activated IP₃R1. Furthermore, co-immunoprecipitation of the ERAD pathway components p97, gp78, and Ufd1 with SPFH2 was slightly enhanced by GnRH treatment, especially at 5 min, as would be expected from the fact that these proteins associate with polyubiquitinated IP₃Rs (Fig. 1A).

RNAi of SPFH2 Inhibits IP₃₃₃ Processing—To investigate the functional significance of the interaction between SPFH2 and activated IP₃₃₃, we used RNAi to “knock down” endogenous SPFH2. We used Rat-1 cells for these studies, as we were previously able to stably knock down p97 in these cells and examine its role in IP₃₃₃ ERAD (39). Here, we utilized a doxycycline-inducible expression system to acutely knock down SPFH2, to avoid possible compensatory mechanisms that might develop in response to prolonged SPFH2 depletion. We screened five siRNAs targeted to different regions of rat SPFH2 mRNA, and maximal knockdown (~75%) was obtained in cells expressing SPFH2si5 (SPFH2si5 cells), as SPFH2 might be more widely involved in the ERAD of cellular substrates. In non-stimulated αT3-1 cells, several proteins with established roles in ERAD (e.g. p97, Hrd1, gp78, Ufd1, and Derlin-1) all co-immunoprecipitated with SPFH2 (Fig. 4A, lane 1). In contrast and as an indicator of the specificity of these interactions, grp94, an ER luminal chaperone, Ufd2, a putative ubiquitin chain elongation factor, and Sec61β, a component of the ER membrane translocation channel, did not co-immunoprecipitate with SPFH2 (Fig. 4A, lane 1). Thus, under resting conditions, a proportion of cellular SPFH2 is linked to ERAD pathway components. Apparently, this proportion is relatively small and the bulk of SPFH2 is free, because cell stimulation causes SPFH2 to associate with IP₃₃₃s with different kinetics from other ERAD pathway components (Fig. 1A). The linkage between SPFH2 and ERAD pathway components is most likely via endogenous substrates undergoing ERAD, because causing the accumulation of these substrates with the proteasome inhibitor bortezomib increased the amount of p97 that co-immunoprecipitated with SPFH2 (Fig. 4B). Importantly, stimulation with GnRH for 0.5 and 5 min substantially increased the amount of IP₃₃₃1 that co-immunoprecipitated with SPFH2, and probing for...
compared with control cells expressing Random siRNA (Random cells) (Fig. 5A). The levels of other related proteins and ubiquitin conjugates were not altered by SPFH2 knockdown (Fig. 5A). Thus, SPFH2 knockdown is specific and does not have a general effect on the conjugation of ubiquitin to cellular substrates.
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Fig. 5B shows that ET1-induced IP$_3$R1 polyubiquitination was reduced ~60% at all times by SPFH2 knockdown. Significantly, polyubiquitination was reduced at the earliest time point measured (5 min), indicating that SPFH2 is involved in the early stages of IP$_3$R1 ERAD, and co-immunoprecipitation of SPFH2 with activated IP$_3$R1 was not detectable in the SPFH2si5 cells, showing that the residual endogenous SPFH2 was not sufficient to maintain normal levels of SPFH2 association with activated IP$_3$R1. Further, and as expected, co-immunoprecipitation of p97 was markedly inhibited (Fig. 5B), most likely because IP$_3$R1 polyubiquitination was reduced and the p97-Ufd1-Npl4 complex binds to polyubiquitinated proteins (21, 22). As expected from these results, ET1-induced IP$_3$R1 down-regulation was markedly inhibited by SPFH2 knockdown (Fig. 5C). To ensure that these effects of SPFH2 knockdown were not due to inhibition of ET1–induced IP$_3$R activation, we measured Ca$^{2+}$ mobilization in response to ET1 and found that it was not inhibited (Fig. 5D). In fact, Ca$^{2+}$ mobilization was slightly increased in the SPFH2si5 cells, most likely because resting IP$_3$R1 levels were elevated ~20% by SPFH2 knockdown (Fig. 5C). To eliminate the possibility that “off-target” effects of SPFH2si5 account for the inhibition of IP$_3$R1 processing, we reintroduced SPFH2 into SPFH2si5 cells via an SPFH2 cDNA construct (SPFH2–5*) that encodes an mRNA resistant to RNAi, demonstrating that HMGR$_{350}$-3HA and CD3δ- HA are degraded much more rapidly than a representative endogenous protein.

We also examined the specificity of the function of SPFH2 using IκBα, a cytosolic protein that is rapidly degraded via the ubiquitin-proteasome pathway, but independently of ERAD, in TNFα-stimulated HeLa cells (57). In contrast to the situation for IP$_3$R1 (Fig. 1A), and for HMGR$_{350}$-3HA and CD3δ- HA (Fig. 6A), SPFH2 did not co-immunoprecipitate with IκBα undergoing polyubiquitination and degradation (Fig. 6C). Further, TNFα-stimulated IκBα polyubiquitination and degradation was not inhibited by SPFH2 knockdown (Fig. 6D). Overall, these data indicate that SPFH2 is specifically involved in the processing of ERAD substrates.

**DISCUSSION**

Endogenous IP$_3$Rs provide a unique tool to study ERAD in mammalian cells, because their activation in response to cell surface receptor stimulation almost instantaneously converts them from stable proteins into ERAD substrates (36). Using IP$_3$R1, other ERAD substrates and RNAi, we discovered that SPFH2 plays an important role in the mammalian ERAD pathway. SPFH2 was found to associate with IP$_3$R1 immediately after its activation, and SPFH2 knockdown inhibited IP$_3$R1 polyubiquitination and subsequent degradation. Likewise, SPFH2 associated with the model ERAD substrates, HMGR$_{350}$-3HA and CD3δ- HA, and SPFH2 knockdown stabilized these proteins. Some SPFH2, most likely a small proportion of the total cellular pool, was also found to be associated with ERAD pathway components in non-stimulated cells, probably via interactions with endogenous substrates undergoing ERAD. Finally, SPFH2 is specifically involved in ERAD, because it did not interact physically or functionally with IκBα undergoing degradation.

SPFH2 appears to be involved in the early steps of ERAD, because it interacts with IP$_3$R1 immediately after its activation and prior to the association of the established ERAD pathway components p97, gp78, and Ufd1, all of which bind to IP$_3$R1 once it has been polyubiquitinated. Further, SPFH2 knockdown inhibited IP$_3$R1 polyubiquitination at the earliest time points measured. Because the bulk of SPFH2 lies within the ER lumen, it is plausible that it interacts with luminal regions of ERAD substrates prior to their exit from the ER, and that it acts as a recognition factor that selects substrates for ERAD. Thus, SPFH2 may function analogously to other ER luminal proteins involved in ERAD substrate recognition, such as Hrd3p and Yos9p in yeast (4, 6, 7) or the EDEM proteins in mammals (27). Alternatively, it remains a formal possibility that SPFH2 directly catalyzes substrate polyubiquitination, perhaps by acting as a novel E3 cofactor.

Given this putative role of SPFH2 in ERAD, it seems somewhat surprising that SPFH2 knockdown did not decrease the levels of generic ubiquitin conjugates in cell lysates (Fig. 5A). There are two possible explanations for this. First, ERAD substrates may represent only a small proportion of the total cellular complement of ubiquitin conjugates, and thus, a decrease in the polyubiquitination of these substrates could go undetected. Alternatively, the amount of SPFH2 that remains after RNAi
25% of normal levels) may be sufficient to process generic ERAD substrates in resting cells but is limiting when IP3Rs are converted into ERAD substrates upon cell stimulation, or when cells are transfected to overexpress HMGR350-3HA or CD3β-HA. Interestingly, an analogous situation occurred when we previously knocked down p97 by 62%; this degree of p97 knockdown did not alter the levels of generic ubiquitin conjugates but did inhibit the processing of IP3Rs when cells were stimulated (39).

How does SPFH2 interact with activated IP3Rs? Conformational changes and the exposure of hydrophobic patches have been proposed to trigger the selection of proteins for ERAD, possibly by allowing for recognition by ERAD pathway components (1, 26). For example, a conformational change in yeast HMGR induced by sterol pathway intermediates appears to render it more susceptible to ERAD (58, 59), and similar changes in mammalian HMGR may allow for the binding of INSIGs (18, 28). To allow Ca2+ to flow from stores within the

**FIGURE 6.** SPFH2 mediates the ERAD of HMGR350-3HA and CD3β-HA but not the polyubiquitination of IκBα. A, lysates from HeLa cells transiently transfected with vectors encoding either HMGR350-3HA or CD3β-HA, were incubated with anti-HA epitope, and immunoprecipitates were probed for SPFH2 or HA epitope, to identify HMGR350-3HA and CD3β-HA. B, HeLa cells transiently transfected with vectors encoding either random siRNA or SPFH2si1 plus SPFH2si5 were transiently transfected again with cDNAs encoding HMGR350-3HA or CD3β-HA. The cells were then incubated with 20 μg/ml cycloheximide for the times indicated, and cell lysates were probed for the indicated proteins. Quantitated data for HMGR350-3HA (n = 3) and CD3β-HA (n = 5) are graphed; *, p < 0.05 comparing results from cells expressing Random or SPFH2si1 plus 5 siRNAs.

C, HeLa cells were treated with 2 ng/ml TNFα for the indicated times, without (-) or with (+) a 30-min preincubation with 1 μM bortezomib, cell lysates were incubated with anti-IκBα, and immunoprecipitates were probed for SPFH2 and IκBα. The rightmost lane contains 1% of the cell lysate used for the immunoprecipitations.

D, HeLa cells were transiently transfected with siRNAs as in A and then treated with TNFα and/or bortezomib as in B, and cell lysates were probed for SPFH2 and IκBα.
ER lumens into the cytosol, IP$_3$R tetramers undergo an as yet undefined structural transition in response to IP$_3$ and Ca$^{2+}$ binding that likely involves a structural change to the pore of the Ca$^{2+}$ channel, which is located in an intraluminal loop between the fifth and sixth transmembrane domains of IP$_3$Rs (30–32). This luminal loop may provide the docking site for SPFH2, because the bulk of SPFH2 resides in the ER lumen, and this loop is already known to mediate interactions between IP$_3$R1 and chromogranins A and B (60) and ERP44 (61). Likewise, SPFH2 may interact with the luminal domains of other ERAD substrates, such as HMGR$^{350}$-3HA or CD36-HA.

A general function for the SPFH domain has yet to be established, and this, together with the limited sequence homology shared between SPFH domains from different proteins, has been proposed to reflect convergent, rather than divergent, evolution of this motif (42). Despite this, SPFH2 and the other better characterized mammalian SPFH domain-containing proteins (flotillins, prohibitins, stomatin, and stomatin-like proteins) do share some similarities. For example, all have hydrophobic stretches at their N termini that appear to play a role in membrane anchoring and directly precede the SPFH domain (49, 50, 53, 54). In addition, they are often found in oligomeric structures within detergent-resistant membranes (DRMs) (47, 51, 53, 54), which are characterized by high concentrations of cholesterol and glycosphingolipids (41, 42). While we were preparing this manuscript, two groups reported SPFH2 to be a component of DRMs. One group reported that SPFH2, which they named erlin-2, is localized to DRMs derived from the ER (62), and the other, that a small fraction of total cellular SPFH2, which they refer to as C8ORF2, was confined to caveolae (63). In both of these reports, however, the majority of cellular SPFH2 was found in the ER, consistent with our results. Conceivably, ER-resident DRMs could play a role in ERAD by recruiting ERAD pathway components, such as SPFH2, to membrane microdomains, thereby facilitating the assembly and spatial regulation of the multiprotein complexes that mediate ERAD.

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