Interaction between Salt-inducible Kinase 2 (SIK2) and p97/Valosin-containing Protein (VCP) Regulates Endoplasmic Reticulum (ER)-associated Protein Degradation in Mammalian Cells

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Salt-inducible kinase 2 (SIK2) is an important regulator of cAMP response element-binding protein-mediated gene expression in various cell types and is the only AMP-activated protein kinase family member known to interact with the p97/valosin-containing protein (VCP) ATPase. Previously, we have demonstrated that SIK2 can regulate autophagy when proteasomal function is compromised. Here we report that physical and functional interactions between SIK2 and p97/VCP underlie the regulation of endoplasmic reticulum (ER)-associated protein degradation (ERAD). SIK2 co-localizes with p97/VCP in the ER membrane and stimulates its ATPase activity through direct phosphorylation. Although the expression of wild-type recombinant SIK2 accelerated the degradation and removal of ERAD substrates, the kinase-deficient variant conversely had no effect. Furthermore, down-regulation of endogenous SIK2 or mutation of the SIK2 target site on p97/VCP led to impaired degradation of ERAD substrates and disruption of ER homeostasis. Collectively, these findings highlight a mechanism by which the interplay between SIK2 and p97/VCP contributes to the regulation of ERAD in mammalian cells.

SIK2 (also known as QIK) is a serine/threonine kinase belonging to the AMPK family, members of which play crucial roles in sensing energy state and stress response regulation. Existing studies have revealed that SIK2 participates in energy metabolism and modulation of cellular gene expression in response to hormones and nutrients. Specifically, SIK2 modulates the efficiency of insulin signal transduction through IRS-1 Ser-794 phosphorylation and confers insulin resistance in diabetic animals (1). Insulin-activated SIK2 phosphorylates and sequestrates the transducer of regulated CREB protein 2 in cytoplasm, thereby inhibiting CREB-dependent gene expression and consequent hepatic gluconeogenesis (2, 3). Furthermore, SIK2 inhibits lipogenesis in both adipocytes and hepatocytes, respectively, through suppressing expression of lipogenic genes PGC-1α and UCP-1 (4) and inhibitory phosphorylation of Ser-89 on p300 whose histone acetyltransferase activity promotes carbohydrate-responsive element-binding protein-mediated fatty acid synthesis (5). Additional functions of SIK2 via the regulation of the CREB coactivator transducer of regulated CREB protein 1/2 have also been reported, including melano-genesis regulation (6), cerebral ischemia-associated neuronal survival (7), and corticotropin-releasing hormone transcription (8). Moreover, SIK2 localizes in centrosome and regulates the distribution of the centrosome linker protein C-Nap1 through Ser-2392 phosphorylation, thus affecting cell growth (9). SIK2 is required for the maturation of autophagosome when proteasome activity is impaired (10). Notably, a previous study on delineating the interactomes of AMPK-related kinases demonstrated that p97/VCP is a unique component of SIK2-containing complexes (11), suggesting that SIK2 may have a unique function in partnership with p97/VCP.

Members of the AAA (ATPases associated with a variety of cellular activities) protein superfamily are ATPases that form stable oligomeric structure (12). In mammalian cells, p97/VCP is a unique component of SIK2-containing complexes (11), suggesting that SIK2 may have a unique function in partnership with p97/VCP.

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†† The abbreviations used are: SIK2, salt-inducible kinase 2; VCP, valosin-containing protein; KD, kinase-dead; CREB, cAMP response element-binding protein; AMPK, AMP-activated protein kinase; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; VIMP, VCP-interacting membrane protein.
SIK2 Regulates p97/VCP-mediated ERAD

Cdc48p, is the most extensively studied member of type II AAA proteins, which possess two ATPase domains. Intact ATPase activity of p97/VCP is required for various cellular processes such as membrane fusion, programmed cell death, cell cycle control, DNA damage response, transcriptional regulation, metabolic modulation, selective autophagy, and ERAD (13–27). During ERAD, p97/VCP forms a complex with Ufd1-Npl4 heterodimer that plays a key role in extracting proteins from ER to the cytosolic proteasomal degradation machinery (23, 28). This process represents a critical determinant for degrading misfolded proteins and unassembled polypeptides of protein complexes in the ER such as the T cell antigen receptor CD3δ subunit (29) as well as regulating the homeostasis of normal ER-resident proteins (30–33). Recent results further suggest that p97/VCP functions as a key mediator of the cross-talk between autophagy and ubiquitin-proteasome pathways (34). Additionally, the functions of p97/VCP appear to be regulated by phosphorylation. Tyrosine phosphorylation of p97/VCP has been linked to transitional ER assembly, cell proliferation, apoptosis, and cell cycle-dependent nuclear localization (35–38). p97/VCP phosphorylated at Ser-784 was also shown to be recruited to sites of DNA double strand breaks (39). Akt was found to phosphorylate p97/VCP at multiple serine residues, triggering the release of ubiquitinated substrate protein(s) from p97/VCP for degradation (40) as well as mediating cell survival signaling (41). However, the molecular mechanism underlying the regulation of p97 in ERAD has yet to be defined.

In this study, we report a novel function of SIK2 in regulating ERAD. SIK2 activity is required for retrotranslocation of misfolded ER lumen protein for ERAD. Furthermore, SIK2 interacts with p97/VCP and confers activation of its ATPase activity and ERAD function through mediating Ser-770 phosphorylation. Taken together, these results extend the known cellular roles of SIK2 to a critical function in ERAD.

EXPERIMENTAL PROCEDURES

DNA Constructs and Mutagenesis—The wild-type His-p97/VCP and mutant AA plasmids were kindly provided by Dr. Tom A. Rapoport (Department of Cell Biology, Harvard Medical School). pDsRed2-ER was a generous gift from Dr. M. F. Chang (National Taiustion University, Taipei, Taiwan). Use of the plasmids encoding wild-type SIK2 (SIK2-WT), kinase-dead mutant SIK2 (SIK2-KD), and shRNAs against human SIK2 was based on a previous report (10). p97/VCP mutants were produced with a site-directed mutagenesis kit (Stratagene, La Jolla) according to manufacturer’s instructions. The following primers were synthesized for creating the mutants from the pcDNA-His-p97 plasmid: p97-S748A, 5'-CCGACGTCTGTGCCGCTGATAATGACATTC-3'; p97-S770A, 5'-GTCGAGGTTTGGCCGCTTCAATGATTC-3'. The mutations were verified by DNA sequencing. The plasmid expressing HA-CD3δ was a gift from Dr. Shengyun Fang (BioMET, University of Maryland). All plasmids were confirmed by sequencing. The pLKO.1 vectors encoding SIK2 shRNA (Clones TRCN0000037495 and TRCN0000037498) as well as lentivirus utilized for SIK2 knockdown and the control virus were from National RNAi Core Facility (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan). The grp78 promoter-based luciferase reporter was constructed by insertion of the PCR fragment containing grp78 promoter sequence spanning from -314 to +246 (42) into the HindIII and BglII sites of pGL3-Basic vector (Promega, Madison, WI). The p5xNFkB-Luc plasmid was from Stratagene.

Antibodies and siRNA—Mouse monoclonal antibody to His tag was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and those against FLAG tag and HA tag were from Sigma-Aldrich. Anti-furin convertase polyclonal antibody was kindly provided by Dr. T. S. Jou (National Taiwan University, Taipei, Taiwan), and mouse anti-calnexin monoclonal antibody was a gift from Dr. F. J. Lee (National Taiwan University, Taipei, Taiwan). Monoclonal antibodies to p97/VCP (clone 4G9), SIK2 (clone 15G10), and α-tubulin (clone 10D8) as well as rabbit antibodies against gigantin, FLAG tag, and β-actin were generated in the laboratory and affinity-purified according to a standard protocol. Rabbit antibodies against phospho-Ser-748 and phospho-Ser-770 of p97/VCP were generated by keyhole limpet hemocyanin-conjugated phosphopeptides ARRVS*DNAIRSC (S* is Ser-748) and QSRFGFS*FRFPSC (S* is Ser-770). siRNA targeting p97/VCP was acquired from Dharmaco (Chicago, IL).

Cell Culture and Transfection—HEK293T and HeLa cells were maintained as described previously (10). Culture of human foreskin fibroblast HS68 cells was done according to the instructions provided by American Type Culture Collection. Calcium phosphate-mediated transfection of HEK293T cells was carried out according to a standard procedure. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Protein Purification, Immunoprecipitation, and Western Blot Analysis—His-p97/VCP was purified using nickel-nitrilotriacetic acid beads (Qiagen, Chatsworth, CA) and further purified on a Superdex 200 HR (10/30) column (Amersham Biosciences) in buffer containing 50 mM Tris/HCl, pH 8.0, 150 mM KCl, 5% glycerol, and 2 mM MgCl2. Immunoprecipitation was performed as described previously (10). Western blot analysis was performed using the indicated antibodies and subsequently visualized using ECL chemiluminescence (PerkinElmer Life Sciences) and x-ray films (Eastman Kodak Co.). Band signals were scanned before being quantified by the software Image Gauge (Fujifilm, Tokyo, Japan).

In Vitro Pulldown Assay—HEK293T cells were transfected with empty vector or plasmid expressing FLAG-SIK2 and harvested 48 h later. The supernatants of the cell extracts were supplemented with NaCl to 900 mM and Triton X-100 to 1% followed by M2 immunoprecipitation for 1 h at 4°C. Immunopurified FLAG-SIK2 was then incubated with affinity-purified His-p97 for another 1 h at 4°C. The precipitants were resolved by SDS-PAGE and visualized by Coomassie Blue staining.

Immunofluorescence Staining and Confocal Microscopy—HeLa cells grown on coverslips were washed with PBS followed by fixing with 4% formaldehyde for 20 min at room temperature. The cells were permeabilized with 0.5% Triton X-100 for 5 min and blocked by 1% BSA before incubation with the indicated antibodies. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen) were used as secondary antibodies. The nucleus
was counterstained with Hoechst. Cells expressing GFP-SIK2 and the red fluorescent ER marker were stained with DAPI immediately after the fixation step. Images were acquired using an inverted confocal microscope (LSM-510, Zeiss, Thornwood, NY) installed with a 63×/numerical aperture 1.4 oil immersion objective lens. For quantification, 5–10 fields were randomly selected from each sample for analysis.

Biochemical Fractionation—HeLa or HEK293T cells were harvested and resuspended in hypotonic buffer (250 mM sucrose, 20 mM Hepes, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and a mixture of protease inhibitors) on ice for 30 min. Cells were disrupted with a Dounce homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min at 4 °C to remove nuclei and further centrifuged at 100,000 × g for 1 h at 4 °C to yield cytosol and membrane (microsomal) fractions. For extraction of peripheral membrane proteins, the membrane fraction was resuspended in 0.1 M Na₂CO₃, pH 11.2 followed by centrifugation at 100,000 × g for 1 h at 4 °C. The membrane fraction was layered onto 0.3–1.8 M sucrose gradient in KEHM buffer (50 mM Hepes, pH 7.5, 50 mM KCl, 10 mM EGTA, 1.92 mM MgCl₂, 1 mM DTT, and a mixture of protease inhibitors). Ultracentrifugation was performed at 40,000 rpm for 3 h at 4 °C in an SW40 Ti rotor (Beckman). Fractions of 1.1 ml were collected from the top to the bottom of the gradient.

In Vitro Kinase and ATPase Assay—The in vitro kinase assay was performed as described previously (10). The ATPase assay was performed by incubating recombinant His-p97/VCP at 37 °C for 30 min in a 15-µl reaction containing 150 mM KCl, 2.5 mM MgCl₂, 50 mM Tris-Cl, pH 8.0, 1 mM cold ATP, and 0.5 μCi of [γ-³²P]ATP. One microliter of the reaction mixture was subjected to thin-layer chromatography using TLC plastic sheets (PEI cellulose F, Merck). Remaining ATP and hydrolyzed free phosphate were separated by thin-layer chromatography and visualized by autoradiography to determine relative levels.

In-gel Tryptic Digestion and Mass Spectrometry—The gel slices containing protein bands were incubated in 2% β-mercaptoethanol and 25 mM NH₄HCO₃ solution for 20 min followed by adding an equal volume of 10% 4-vinylpyridine, 50% acetonitrile, and 25 mM NH₄HCO₃ solution and incubated for another 20 min. Each processed gel piece was digested overnight by 0.2 μg of trypsin (Promega) at 37 °C. The supernatant of the digestion mixture was collected and analyzed using an LTQ-FT (linear quadrupole ion trap-Fourier transform ion cyclotron resonance) mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a nanoelectrospray ion source (New Objective, Inc., Woburn, MA), an Agilent 1100 Series binary high performance liquid chromatography pump (Agilent Technologies, Palo Alto, CA), and a Famos autosampler (LC Packings, San Francisco, CA).

Statistical Analysis—The data were analyzed with Student’s t test. Data are mean ± S.D. p values <0.05 were considered significant.

RESULTS

Identification of p97/VCP as an Interacting Protein of SIK2—To further investigate the cellular functions and regulation of SIK2, we searched for SIK2-associated proteins using FLAG-SIK2 immunoprecipitation followed by mass spectrometric analysis. We identified p97/VCP from the immunoprecipitates as a candidate interacting partner (Fig. 1A). We next verified the interaction between the endogenous SIK2 and p97/VCP through co-immunoprecipitation analysis using an anti-SIK2 monoclonal antibody, 15G10 (Fig. 1B). To further confirm their direct physical interaction, we performed pulldown assays by incubating purified recombinant His-p97/VCP and recombinant FLAG-SIK2 in the presence of anti-FLAG (M2)–agarose beads and subsequently demonstrated a specific pulldown of His-p97/VCP (Fig. 1C). Moreover, co-immunoprecipitation experiments with SIK2 truncated fragments revealed that SIK2 interacts with p97/VCP through its C-terminal sequences (Fig. 1D).

The functions of p97/VCP in ERAD and membrane fusion are known to be associated spatially with ER membrane. The direct interaction between SIK2 and p97/VCP implies that SIK2 may also be localized to ER membrane with overlapping functional roles. To first demonstrate an ER localization of
SIK2 Participates in ERAD through Regulating Retrotranslocation of Misfolded ER Lumen Proteins—The interaction and ER co-localization of p97/VCP and SIK2 prompted us to investigate the possible involvement of SIK2 in p97/VCP-mediated ERAD. To this end, we examined the degradation of recombinant CD3ε, a known ERAD substrate. CD3ε accumulated in cells upon overexpression of ATPase-deficient p97/VCP (p97-AA; Fig. 3A, lanes 5, 7, and 9), consistent with the notion that p97/VCP ATPase activity is required for disposing ERAD substrates (43). Interestingly, the level of recombinant CD3ε was drastically decreased when SIK2-WT was expressed (lanes 2 and 6) but remained comparable with vector control (lane 1) in the presence of SIK2-KD (lanes 3 and 8). However, the decrease of CD3ε in response to SIK2-WT expression was alleviated by co-expression of p97-AA (lane 7). These results thus suggest that efficient ERAD was mediated by the ATPase activity of p97/VCP and possibly relies on the kinase activity of SIK2.

Down-regulation of endogenous SIK2 by shRNA also resulted in the accumulation of CD3ε (Fig. 3B), further supporting the link between SIK2 and ERAD. To confirm that the reduction of CD3ε by SIK2 overexpression could be attributed to enhanced ERAD, cells co-expressing CD3ε with SIK2-WT or SIK2-KD were treated with cycloheximide, and the kinetics of degradation was followed. As shown in Fig. 3, C and D, CD3ε degradation was clearly enhanced by the overexpressed SIK2-WT when compared with vector control, SIK2-KD (Fig. 3C), or SIK2 knockdown (Fig. 3D). These results suggest that SIK2 facilitates p97/VCP-mediated CD3ε degradation by ERAD. Additionally, suppressed degradation of endogenous 3-hydroxy-3-methylglutaryl coenzyme A reductase, another ERAD substrate (44), was observed in SIK2 knockdown Hs68 fibroblast cells (Fig. 3E), further confirming that SIK2 is required for the turnover of ERAD substrates.

Because ATPase activity of p97/VCP is essential for the retrotranslocation of misfolded proteins from ER to cytosol during ERAD, the extent of which can be assessed by the residual levels of CD3ε in the microsomal fraction (as described under “Experimental Procedures”), we next examined the roles of SIK2 in this functional aspect. As a control, we observed microsomal CD3ε accumulation when p97-AA mutant was expressed (Fig. 4A, left panel). A reduced level of microsomal CD3ε in the presence of SIK2-WT, but not in the SIK2-KD-expressing cells, attests to the role of SIK2 kinase activity in regulating the ret-

SIK2, we performed immunofluorescence staining of cells co-transfected with an ER marker vector (pDsRed2-ER) and/or recombinant SIK2. Both endogenous and recombinant SIK2 (GFP-SIK2 and FLAG-SIK2) exhibited cytoplasmic distribution and partially associated with ER (Fig. 2A, top). The cytoplasmic staining pattern of SIK2 was comparable with that of p97/VCP (Fig. 2A, bottom). Next, to verify whether SIK2 exists in intracellular membrane structures, we fractionated post-nuclear supernatant and probed for SIK2 by immunoblotting. Both recombinant SIK2 and endogenous SIK2 were detected in the membrane fraction (Fig. 2B). Furthermore, SIK2 was extractable from the membrane fraction by alkaline solution, suggesting a peripheral association with ER membrane (data not shown). We further fractionated the membrane fraction in a sucrose gradient to resolve the submembrane distribution of SIK2 and p97/VCP. The results revealed that they shared similar distribution patterns with clear enrichment in cytosol (i.e. fractions 2 and 3) and calnexin-positive ER membrane (fraction 8) (Fig. 2C). In contrast, SIK2 displayed a different distribution from the Golgi marker furin convertase, which was enriched in fractions 4 and 5. Taken together, these data illustrate a partial co-localization between SIK2 and p97/VCP that overlaps preferentially with the ER membrane.

FIGURE 2. Co-localization of SIK2 and p97/VCP in the ER membrane. A, top, HeLa cells grown on coverslips were transfected with DsRed2-ER (to visualize ER) alone (lower) or together with GFP-SIK2 (upper). Endogenous SIK2 was probed by mouse anti-SIK2 antibody (15G10). Bottom, HeLa cells were co-transfected with FLAG-SIK2 and His-p97 plasmids followed by immunofluorescence staining. The images were acquired using a confocal microscope. B, subcellular fractionation of overexpressed FLAG-SIK2 (left panel) or endogenous SIK2 (right panel). These fractions were analyzed by Western blotting. Giantin and α-tubulin are markers for the membrane (Mem) and cytosolic (Cyt) fractions, respectively. C, the membrane fraction derived from HEK293T cells was separated by ultracentrifugation in a 0.3–1.8 M sucrose gradient. 10 × 1 ml fractions were then collected and subjected to Western blot analysis using the indicated antibodies. IB, immunoblotting.
rotranslocation process (Fig. 4A, left panel). Moreover, down-regulation of endogenous SIK2 resulted in the accumulation of CD3\(\alpha\)/H9254 in the membrane fraction as shown by both Western blot (Fig. 4A, right panel) and immunofluorescence staining analyses (Fig. 4, B–D). Collectively, these results implicate the SIK2-p97/VCP interaction as well as the kinase activity of SIK2 in facilitating efficient retrotranslocation and removal of substrates during ERAD.

Given the essential role of SIK2 in ERAD substrate retrotranslocation, we hypothesize that SIK2 may also be required for the maintenance of ER function. To this end, the effect of SIK2 depletion on the ER unfolded protein response and ER overload response was examined by assaying the grp78 promoter activity and the activation of NF-\(\kappa\)B, respectively (45, 46). Because VCP-interacting membrane protein (VIMP/SelS) is a known positive regulator of ERAD through its interaction...
with p97/VCP (28), knockdown of VIMP was included as a control. As shown in Fig. 4E, depletion of SIK2 up-regulated NF-κB and the grp78 promoter activity, suggesting that SIK2 is indispensable for maintaining ER homeostasis. Surprisingly, depletion of SIK2 led to a greater extent of ER stress than VIMP knockdown. Additionally, we observed that an increased level of p97/VCP was co-immunoprecipitated with VIMP when SIK2 was down-regulated (Fig. 4F), whereas knockdown of VIMP enhanced the interaction between SIK2 and p97/VCP (data not shown), implying that SIK2 and VIMP might form
distinct complexes with p97/VCP in a competitive manner. Furthermore, consistent with their distinct roles in p97/VCP-mediated ERAD, CD3δ accumulation induced by VIMP knockdown was reversed by SIK2 overexpression (Fig. 4G, lane 6). Unexpectedly, overexpression of VIMP resulted in CD3δ accumulation in the control and SIK2 knockdown cells (Fig. 4G, lanes 2 and 4). To resolve this discrepancy, we thus performed a time course experiment to trace the protein level of CD3δ/H9254 in VIMP-overexpressing cells. The results showed a decrease in CD3δ levels at 20 h after transfection but a subsequent accumulation at 40 h after transfection (Fig. 4H, lanes 10 and 12), signifying that VIMP is not sufficient for ERAD substrate processing upon long term ER stress. In contrast, in cells expressing SIK2-WT, the level of CD3δ remained low irrespective of the length of transfection (Fig. 4H, lanes 4-6), providing additional evidence for the different roles of SIK2 and VIMP in ERAD. Taken together, these data indicate that SIK2 may play more prominent roles in p97/VCP-mediated ERAD than VIMP.

SIK2 Phosphorylates p97/VCP on Ser-770—Next, to determine whether a kinase-substrate relationship exists between SIK2 and p97/VCP, we performed an in vitro kinase assay on FLAG-SIK2 M2 immunocomplexes. Two distinct bands corresponding to wild-type FLAG-SIK2 and endogenous p97/VCP were detected by autoradiography (Fig. 5A, lanes 2 and 5), indicating that both SIK2 and p97/VCP were phosphorylated. The radioactive signals became undetectable when similar experiments were performed with kinase-dead mutant SIK2 (Fig. 5A, lanes 3 and 6). These observations thus suggest that phosphorylation of SIK2 and p97/VCP depends on SIK2 kinase activity. The substrate specificity of SIK2 toward p97/VCP was further corroborated by the absence of phosphorylation on the co-precipitated Hsp70.

To further delineate the regulatory consequences of the SIK2-mediated phosphorylation of p97/VCP, we next purified endogenous p97/VCP protein by co-immunoprecipitation with wild-type or kinase-deficient SIK2 and subsequently profiled its phosphorylation using a mass spectrometric approach. Two phosphorylation sites, Ser-748 and Ser-770, were detected as candidate target sites and further confirmed by MS2 and MS3 analysis of the corresponding phosphopeptides (supplemental Fig. S1). Based on the quantity of the phosphorylated peptides from selected ion chromatograms, the phospho moieties on Ser-748 and Ser-770 were evident on SIK2-WT-associated p97/VCP but much less evident on SIK2-KD-associated p97/VCP (supplemental Table S1 and Fig. S2). An immunoblotting assay using antibodies against phosphorylated p97/VCP Ser-748 and phosphorylated p97/VCP Ser-770 showed similar phosphorylation profiles (data not shown). These results therefore imply
that SIK2 kinase activity is likely responsible for phosphorylating p97/VCP on these two residues. To further examine such hypothesis, an in vitro kinase assay was next performed using purified recombinant SIK2 and p97/VCP. We found that both p97/VCP-WT and p97/VCP-S748A were similarly phosphorylated by SIK2-WT (Fig. 5B, lanes 2 and 4). In contrast, the level of SIK2-mediated phosphorylation of p97/VCP-S770A or p97/VCP-S748A/S770A was significantly reduced (Fig. 5B, lanes 5 and 6), indicating that p97/VCP Ser-770 is directly phosphorylated by SIK2, whereas p97/VCP Ser-748 may likely be an indirect target. However, the phosphorylation state of p97/VCP Ser-770 did not seem to alter the subcellular localization of p97/VCP as demonstrated by immunofluorescence staining of FLAG-p97/VCP and FLAG-p97/VCP-S770A (data not shown).

In addition to the in vitro assay, we further inspected Ser-770 phosphorylation on the endogenous SIK2-associated p97/VCP in cells. Consistent with the results of in vitro kinase assay, p97/VCP Ser-770 phosphorylation was highly enhanced upon association with SIK2-WT (Fig. 5C, lanes 1 and 2). Interestingly, Ser-770-phosphorylated p97/VCP was also enriched in the co-immunoprecipitates of SIK2-KD but to a lesser extent than SIK2-WT (Fig. 5C, lanes 3 and 4), suggesting that the kinase activity of SIK2 is not required for its association with Ser-770-phosphorylated p97/VCP but may contribute to its level and preferential association with the kinase. Moreover, enrichment of Ser-770-phosphorylated p97/VCP was observed in the endogenous SIK2 immunoprecipitates (Fig. 5D, lanes 3 and 4). Notably, association of phosphorylated p97/VCP with SIK2 was stimulated by CD36 overexpression, which induced SIK2 activity (Fig. 5D, lanes 1 and 2). Taken together, these data are in line with the notion that the accumulation of ERAD substrate activates SIK2, leading to elevated Ser-770 phosphorylation on SIK2-associated p97/VCP.

**SIK2-mediated Stimulation of p97/VCP ATPase Activity and Its ERAD Function by Ser-770 Phosphorylation**—Having demonstrated the phosphorylation of p97/VCP by SIK2, we then sought to investigate whether the ATPase activity of p97/VCP is modulated by SIK2. In an initial ATPase assay in which purified p97/VCP was preincubated with SIK2-WT or SIK2-KD, an elevated ATPase activity of p97/VCP was observed upon preincubation with SIK2-WT but not with SIK2-KD (data not shown). To further specify the correlation between Ser-770 phosphorylation and ATPase activity of p97/VCP, the S770A and S770D mutants were also assessed by ATPase assay. The results consistently showed that preincubation with SIK2-WT led to a considerably higher phosphorylation level as well as ATPase activity of p97/VCP as compared with SIK2-KD (Fig. 6, A, lanes 1 and 4, and B, left). Moreover, for the phosphodefective p97/VCP-S770A mutant, phosphorylation by SIK2 and the up-regulation in ATPase activity were moderately impaired (Fig. 6, A, lanes 2 and 5, and B, middle), further indicating that

[FIGURE 6. Phosphorylation of p97/VCP by SIK2 enhances its ATPase activity. A, in vitro phosphorylation of p97/VCP was performed by incubating affinity-purified His-p97/VCP (WT, S770A, or S770D) with FLAG-SIK2 (WT or KD) in the presence of 50 μM ATP. Following the reaction, an ATPase assay was carried out. Upon incubation, 1 μl of the reaction mixture was subjected to TLC and subsequently autoradiography. Pi and ATP, respectively, denote the hydrolyzed free radioactive phosphate and the remaining [γ-32P]ATP. One representative result (of three reproducible experiments) is shown. B, quantitative results of the relative ATPase activities shown in A measured from triplicate experiments. Error bars represent S.D. *, p < 0.01 for three independent experiments. IB, immunoblotting.]
SIK2-mediated Ser-770 phosphorylation closely correlates with elevation of p97/VCP ATPase activity. Of note, minimal Ser-770 phosphorylation of p97/VCP was detected in the presence of SIK2-KD (Fig. 6A, lane 4) possibly due to endogenous SIK2 activity in the cells from which His-p97/VCP was overexpressed and purified. The phosphorylated Ser-770 detected on p97/VCP-S770A and p97/VCP-S770D (Fig. 6A, lanes 2 and 3) could also be attributed to endogenous p97/VCP, which exists as a hexamer with the isolated recombinant p97/VCP. Intriguingly, the attributes of the presumably phosphomimetic p97/VCP-S770D mutant were found to be comparable with the p97/VCP-S770A mutant and remained unaltered irrespective of SIK2-WT or SIK2-KD pretreatment, suggesting that such residue change failed to mimic the phosphorylated state for this protein (Fig. 6, A, lanes 3 and 6, and B, right).

To address the functional relevance of p97/VCP Ser-770 phosphorylation in ERAD, we performed knockdown of endogenous p97/VCP with siRNA and complemented with wild-type or the presence of co-expressed SIK2-WT. As shown in Fig. 7A, when compared with the wild-type p97/VCP (lanes 3 and 5), expression of p97/VCP-S770A resulted in accumulation of CD3δ (lanes 4 and 6). Furthermore, the kinetics of cycloheximide chase experiments clearly showed that, even in the presence of SIK2-WT, expression of this non-phosphorylatable form of p97/VCP retarded kinetics of CD3δ degradation (Fig. 7B). Intriguingly, overexpression of SIK2-KD resulted in much
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slower turnover of CD3δ than co-expression of p97-S770A and SIK2-WT, revealing that there may be additional SIK2-mediated mechanisms underlying ERAD regulation. Taken together, these results support the notion that the interaction between SIK2 and p97/VCP and in part the Ser-770 phosphorylation-dependent ATPase activity up-regulation are responsible for the removal of CD3δ by ERAD.

DISCUSSION

The ATPase activity of p97/VCP and its regulation are central to the process of ERAD. In this study, we demonstrated that SIK2 serves as an additional layer of ERAD regulation through its interaction with p97/VCP (partly attributed to its phosphorylation). Several lines of evidence support this scenario. 1) SIK2 exhibits physical and spatial association with p97/VCP (Figs. 1 and 2). 2) SIK2 facilitates the removal of ERAD substrates in a kinase-dependent manner, and knockdown of SIK2 disturbs ER homeostasis (Figs. 3 and 4). 3) SIK2 enhances the ATPase activity of p97/VCP through targeting the Ser-770 residue (Fig. 6). Consequently, this phosphorylation is likely responsible, at least in part, for stimulating the known functions of p97/VCP in retrotranslocation of ubiquitinated proteins from the ER to cytosol and degradation of ERAD substrates (Fig. 7). Collectively, these results strengthen a novel function of SIK2 in p97/VCP-mediated ERAD and thus extend the spectrum of known cellular roles of SIK2.

The Cdc48, Npl4, and Ufd1 complex in yeast provides the driving force for ERAD substrate retrotranslocation (47, 48). In mammalian cells, in addition to p97/VCP-Npl4-Ufd1 complex, VIMP is an additional important player (28). In this study, we have demonstrated that SIK2 may be even more critical than VIMP for facilitating p97/VCP ATPase-mediated retrotranslocation of ERAD substrate. These results suggest that, although the basal machinery of ERAD is evolutionarily conserved, additional layers of quality control for ERAD are evident in mammalian cells.

Although two residues on p97/VCP were identified as candidate substrate sites for SIK2, functional interaction analyses as well as in vitro kinase assay unequivocally demonstrated that Ser-770, rather than Ser-748, is the direct target of SIK2. Phosphorylation of p97/VCP Ser-748 is likely an indirect consequence of SIK2 activation and may thus require additional kinase(s)/protein(s). Furthermore, as no discernible change in the degradation of CD3δ was observed in cells co-transfected with SIK2-WT and p97/VCP-S748A as opposed to SIK2-WT and p97/VCP-S770A (data not shown), it remains a formal possibility that p97/VCP Ser-748 phosphorylation may have roles in other cellular processes.

As a multifunctional protein, it is clear that binding sites primarily on the N- and C-domains of p97/VCP coordinate the diverse roles of p97/VCP by interacting with a myriad of cofactors. SIK2, the new addition to this group of factors, associates with p97/VCP via its unusually glutamine-rich (~22% in content) C-terminal region (Fig. 1D). In addition, overexpression of SIK2 N-terminal fragment alone was able to induce p97/VCP Ser-770 phosphorylation to an extent similar to the full-length SIK2, revealing that SIK2 interacts with and phosphorylates p97/VCP through distinct domains and that the C-terminal p97/VCP-interacting region of SIK2 may be dispensable for Ser-770 phosphorylation (Fig. 1D and data not shown). A unifying theme among known p97/VCP cofactors is the ubiquitin-binding competence and possible involvement in ubiquitination-dependent processes (49). Although a ubiquitin moiety binding has not yet been ascribed to SIK2, the presence of a ubiquitin-associated domain strongly insinuates a cooperative sequestering of polyubiquitinated proteins by SIK2 and p97/VCP under normal and/or stress conditions. Such functional relevance at the protein quaternary structure level may be further elucidated by biophysical characterization.

Other than ERAD, p97/VCP has been implicated in many aspects of cellular homeostasis, including membrane fusion after mitosis, processing of protein aggregates, and cell cycle control (16–24). These functional aspects may be mediated by its ability to interact with and control the fate of ubiquitinated proteins. Additionally, p97/VCP was proposed as a sensor for aggregated proteins and reportedly implicated in various neurodegenerative disorders such as Parkinson, Alzheimer, and polyglutamine diseases (50, 51). In line with its crucial roles in autophagy, mutations in p97/VCP are also linked to human multisystem disorders, such as inclusion body myopathy, Paget disease of the bone, and frontotemporal dementia (52–54). Thus, p97/VCP and its interacting cofactors can target protein degradation by both the ubiquitin-proteasome system and autophagy pathways (34). Our recent work has revealed that SIK2 is required for autophagy when proteasomal activity is impaired (10). The current results, demonstrating that the interaction between SIK2 and p97/VCP regulates ERAD, supports the possibility that SIK2, like p97/VCP, may serve as a key regulator in the ubiquitin-proteasome system and autophagy. Furthermore, this regulatory network may have significant implications in cancer biology as previous reports have shown that elevated p97/VCP levels correlate with metastasis of several types of cancers (55–58) and that SIK2 is required for cancer cell growth (9). Collectively, these observations strengthen the notion that SIK2 is necessary for the proper functions and integrity of the ubiquitin-proteasome system and autophagy, which in turn are critical for cancer cell proliferation and survival.

The physiological relevance of SIK2 in ERAD may be further considered in the broader context of glucose and fatty acid homeostasis to which AMPK family members are intimately linked. As a member of the energy-sensing AMPK family (59), SIK2 has been demonstrated to be activated, similarly to other AMPK-related kinases, through LKB1-mediated phosphorylation in response to an increase in the AMP/ATP ratio or under low glucose conditions (60). However, the elevated AMP/ATP ratio may not play any role in SIK2-regulated ERAD as demonstrated by failure of 5-aminoimidazole-4-carboxamide 1-β-d-ribofuranoside to activate SIK2 and p97/VCP phosphorylation (data not shown). We have also observed that SIK2 is highly induced in tissues under stress conditions (e.g. hypoxia) and in cancer cells. Because a deficit in cellular energy may trigger

4 F.-C. Yang, Y.-H. Lin, W.-H. Chen, J.-Y. Huang, H.-Y. Chang, S.-H. Su, H.-T. Wang, C.-Y. Chiang, P.-H. Hsu, M.-D. Tsai, B. C.-M. Tan, and S.-C. Lee, unpublished results.
protein misfolding and consequently induce the unfolded protein response (61), SIK2 activity may thus play important roles in relieving ER stress by facilitating ERAD. The pathophysiological significance of the role of SIK2 in linking cellular energy status and metabolism to protein degradation remains to be elucidated.

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