Serum-inducible kinase (SNK) is a member of polo-like kinases that serve as regulators of multiple events during cell division. Rapid changes in the activity and abundance of SNK were reported after the serum stimulation and after the activation of synaptic transmission in the brain. Yet the detailed mechanisms that control the level of SNK protein have not been fully elucidated. In this report, we show that the RING-H2 domain of hVPS18 (human vacuolar protein sorting 18) has a genuine ubiquitin ligase (E3) activity. Using the yeast two-hybrid screening, we identify SNK as a candidate substrate of hVPS18. The half-life of SNK is increased in HeLa cells that down-regulated hVPS18 by lentivirus-mediated small hairpin RNA interference. Furthermore, the delayed entry into S phase is observed in HeLa cells overexpressing hVPS18. These results suggest that hVPS18 may play an important role in regulation of SNK activity through its ubiquitin ligase.

The polo-like kinases are a family of serine/threonine protein kinases that include mammalian polo-like kinase (Plk1), SNK (Plk2), fibroblast growth factor (Plk3), Sak (Plk4), Caenorhabditis elegans Plc1–3, Xenopus laevis Plx1, Drosophila polo, fission yeast Plo1, and budding yeast Cdc5. Genetic and biochemical evidence in various organisms indicates that polo-like kinases play pivotal roles in regulating many cell-cycle dependent processes such as centrosome maturation and separation, mitotic entry, metaphase to anaphase transition, mitotic exit, and cytokinesis. A number of studies suggest that the kinase activity is regulated by a variety of post-translational processes, including phosphorylation and ubiquitin conjugation.

The mammalian homologues of yeast Class C type of vacuolar protein sorting (VPS) proteins appear to control the fusion events of late endosomes and lysosomes that are the major pathway of the intracellular proteins. Despite the recent investigations of the Class C VPS in the membrane traffic of the yeast and mammals, the molecular mechanisms remain undefined. In this report, we demonstrate that hVPS18 is a genuine ubiquitin ligase and search for the ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The RING-H2 finger is a zinc binding domain with an octet of cysteines and histidines with a defined space representing the largest class of ubiquitin ligases to date. For instance, mammalian genomes encode hundreds of RING finger proteins, and it has not yet been clear whether all of these proteins have E3 activity or not.

In this regard, a number of molecules are waiting for characterization that may help us to understand how the intracellular protein levels are controlled. Despite the recent investigation of the Class C VPS in the membrane traffic of the yeast and mammals, the molecular mechanisms remain undefined. In this report, we demonstrate that hVPS18 is a genuine ubiquitin ligase and search for ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). We screened the yeast two-hybrid screen, the yeast strain AH109 was transformed sequentially with pGBK7-hVPS18, as a bait, and a cDNA library of the rat brain (BD Biosciences Clontech) using the lithium acetate method. We screened $2 \times 10^8$ transformants on the plate containing synthetic dropout medium lacking histidine, tryptophan, and leucine. Positive clones were selected on 5 mM 3-aminoimidazole-containing medium lacking leucine, tryptophan, and histidine and verified with a filter assay for $\beta$-galactosidase activity. The prey plasmids were recovered and sequenced. The full-length SNK was isolated by reverse transcription-PCR from the rat brain cDNA. We assessed the interaction of hVPS18 and rat SNK in AH109 cells transformed sequentially with pGBK7-hVPS18 (full-length or $\Delta$RING) and pGADT7 containing SNK or clone A12-1 by quantifying $\beta$-galactosidase activity.
Ubiquitylation of SNK by hVPS18

Plasmid Construction—Full-length and various truncated SNK cDNAs were subcloned into the following vectors: the pGEX-4T2 (Amersham Biosciences) prokaryotic expression vector for the production of GST-tagged fusion proteins; the pCMV-HA, pCMV-FLAG, and pCMV-Myc mammalian expression vectors (BD Biosciences Clontech) for the production of HA-tagged, FLAG-tagged, and Myc-tagged fusion proteins. Myc- and HA-tagged hVPSs (hVPS11, hVPS18, hVPS18ΔRING, hVPS16, and hVPS33a) mammalian expression vectors were prepared as described previously (6). The full-length hVPSs (hVPS11, hVPS18, and hVPS16) were subcloned into pFastBacHTb insect expression vector (Invitrogen) to generate His-tagged hVPSs.

Expression and Preparation of Recombinant Proteins—Ubiquitin-conjugating enzymes (E2s) were expressed in E. coli strain BL21-AI (Invitrogen) and lysed in a buffer containing phosphate-buffered saline, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture (Roche Molecular Biochemicals). The supernatant, after centrifugation at 14,000 × g for 10 min, was used for the ubiquitylation reaction. Full-length and various truncated mutants of GST-SNK were expressed in E. coli strain BL21-AI, and the recombinant proteins were purified using glutathione-Sepharose 4B beads (Amersham Biosciences) in phosphate-buffered saline, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor mixture, and ammonium acetate 250 mM NaCl. The supernatant, after centrifugation, was subjected to SDS-PAGE and detected by Western blot analyses using anti-SNK antibody, or anti-hVPS18 antibody.

In Vivo and in Vitro Ubiquitylation Assay—Myc-tagged hVPS18, hVPS11, hVPS18, FLAG-tagged SNK, and HA-tagged ubiquitin were co-transfected to COS7 cells using FuGENE 6. Transfected cells were treated with 30 μM MG132 for 3 h at 16 h post-transfection. Immunoprecipitation was performed by using anti-FLAG (M2)-agarose, and conjugated HA-ubiquitin was detected by Western blot analyses using anti-HA antibody.

Establishment of HeLa Cell Lines—Up-regulates/Down-regulates hVPS18 by Lentivirus Vector—The lentivirus containing full-length of hVPS18 or short hairpin RNA (shRNA) interference was raised by the method previously described (13). Briefly, synthetic oligonucleotides (top-strand: 5′-GATCCCCAGGTTGTACCACCTTGCAAAAGCTGGTGCTTGCGCTTTTGGAAAGATGGGCACTTTTTTGGAAATTT-3′; bottom-strand: 5′-CTAGATTTCAAAAAAGTGGCCATCTTCCAAACGCGGACAGCACACCGTCTGGCAAGATGGGCACTTTTTTGAGG-3′; underline, loop sequence) were annealed and ligated into the BgII-XbaI site of pENTR4-H1. The full length of hVPS18 cDNA was ligated into the EcoRI-Xhol site of pENTR3C. Then either pENTR4-H1-hVPS18-shRNA or pENTR3C/hVPS18 was incubated with pLent6/V5 DEST (Invitrogen) in the presence of Gateway LR Clonase for plasmid recombination. To obtain recombinant lentivirus, the Clonase-recombinated plasmid was co-transfected with packaging construct (pCAG-GFP), and vesicular stomatitis virus G glycoprotein-expressing, Rev-expressing construct (pCMV-VSV-G-RSV) to HEK293T cells. The titer of virus was determined by measuring the amount of human immunodeficiency virus, type 1 p24 gag antigen using an enzyme-linked immunosorbent assay kit (PerkinElmer Life Sciences).

Measurement of Protein Phosphorylation/Degradation—U-254 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified incubators with 5% CO2 at 37 °C. The cells were transfected with various plasmids by FuGEnE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Antibodies—His-tagged full-length rat SNK was expressed in SF9 cells. The protein was purified by ProBond metal affinity resin and used as immunogens in Wistar rats. The affinity purification using His-SNK coupled to a HiTrap NHS-activated column (Amersham Biosciences) was performed according to the manufacturer’s instruction. The rabbit anti-SNK polyclonal antibody was kindly provided by Dr. M. H. Sheng. Anti-hVPS antibodies (hVPS11, hVPS16, and hVPS18) were prepared as described previously (6). Other antibodies used were as follows: mouse monoclonal anti-Myc antibody (9E10, Roche Molecular Biochemicals), rabbit monoclonal anti-HA antibody (3F10, Roche Molecular Biochemicals), mouse monoclonal anti-FLAG (M2)-agarase (Sigma), rabbit polyclonal anti-ubiquitin antibody (Sigma), and mouse monoclonal anti-α-tubulin antibody (B-5-1-2, Sigma).

Immunoprecipitation—For immunoprecipitation, the cells were lysed in modified radioimmune precipitation assay buffer (50 mM Tris, pH 7.6, 250 mM NaCl, 1% Triton X-100, 3 mM EDTA) supplemented with Complete protease inhibitor mixture. Total cell lysates were centrifuged at 10,000 × g for 10 min, and the protein concentration of the supernatants was determined. Identical amounts of the protein from each sample were precleared by incubation with protein A/G-Sepharose 4 fast flow (Amersham Biosciences) for 30 min at 4 °C. After the removal of protein A/G-Sepharose by brief centrifugation, the solution was incubated with 2 μg of monoclonal anti-Myc antibody, monoclonal anti-HA antibody, or control IgGs at 4 °C overnight. Immunoprecipitation of the antigen-antibody complex was accomplished by adding 40 μl of protein A/G-Sepharose for 1 h at 4 °C. Sepharose-bound proteins were solubilized in 40 μl of SDS sample buffer. Samples were boiled and separated by SDS-PAGE, and Western blot analyses were performed.

In Vitro Full-down Assay—Full-length and various truncated mutants of GST-SNK were immobilized on glutathione-Sepharose 4B beads and equilibrated with wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 3 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride) for three times. The resin was incubated with His-tagged hVPSs at 25 °C for 30 min and washed with the wash buffer for three times. The resin was subjected to SDS-PAGE, and Western blot analyses were performed by either anti-hVPS18 antibody, anti-hVPS11 antibody, or anti-hVPS16 antibody.

An in vitro ubiquitylation assay was performed as described previously (12). GST-SNK full-length was mixed with yeast E1 (500 ng, Boston Biochem), E2-enzyme mixture (Boston Biochem), or UbcH4, ubiquitin (10 μg, Boston Biochem) and His-hVPS18. The mixture was incubated at 25 °C for 30 min in the presence of 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 2 mM dithiothreitol, and 2 mM ATP in a 50-μl volume. After incubation, the mixture was subjected to SDS-PAGE and detected by Western blot analyses using anti-GST antibody, anti-ubiquitin antibody, or anti-hVPS18 antibody.

Establishment of HeLa Cell Lines—Up-regulates/Down-regulates hVPS18 by Lentivirus Vector—The lentivirus containing full-length of hVPS18 or short hairpin RNA (shRNA) interference was raised by the method previously described (13). Briefly, synthetic oligonucleotides (top-strand: 5′-GATCCCCAGGTTGTACCACCTTGCAAAAGCTGGTGCTTGCGCTTTTGGAAAGATGGGCACTTTTTTGGAAATTT-3′; bottom-strand: 5′-CTAGATTTCAAAAAAGTGGCCATCTTCCAAACGCGGACAGCACACCGTCTGGCAAGATGGGCACTTTTTTGAGG-3′; underline, loop sequence) were annealed and ligated into the BgII-XbaI site of pENTR4-H1. The full length of hVPS18 cDNA was ligated into the EcoRI-Xhol site of pENTR3C. Then either pENTR4-H1-hVPS18-shRNA or pENTR3C/hVPS18 was incubated with pLent6/V5 DEST (Invitrogen) in the presence of Gateway LR Clonase (Invitrogen) for plasmid recombination. To obtain recombinant lentivirus, the Clonase-recombinated plasmid was co-transfected with packaging construct (pCAG-GFP), and vesicular stomatitis virus G glycoprotein-expressing, Rev-expressing construct (pCMV-VSV-G-RSV) to HEK293T cells. The titer of virus was determined by measuring the amount of human immunodeficiency virus, type 1 p24 gag antigen using an enzyme-linked immunosorbent assay kit (PerkinElmer Life Sciences).

The recombinant lentivirus (titer: 5 × 106 IU/ml) was infected to HeLa cells with 10 μg/ml Blasticidin S (Invitrogen) to select the virus-infected cells. The Blasticidin S-resistant colony was identified and expanded to establish the HeLa cell lines that constitutively over-express hVPS18 (HeLa-hVPS18(OE)) or constitutively knock-down hVPS18 by shRNA interference (HeLa-hVPS18(shRNA)). To confirm the expression level of hVPS18 and SNK in the established HeLa cell lines, Western blot analyses and immunocytochemistry were performed as described previously (6).

[35S]Methionine and [35S]Cysteine Pulse-chase Analysis—Three lines of HeLa cells (Control, hVPS18(OE), and hVPS18(shRNA)) were subjected to the in vitro pulse-chase analyses using [35S]methionine/
RESULTS

RING-H2 Domain of hVPS18 Has a Ubiquitin Ligase Activity—It has been shown that hVPS18, a member of Class C VPS protein, has a RING-H2 finger domain in its C-terminal region (6). To examine whether hVPS18 has a ubiquitin ligase activity, we performed in vitro ubiquitylation assay using an E. coli lysate as an anonymous substrate. Because Nedd4 has a HECT (homologous to E6-associated protein carboxy terminus) type ubiquitin ligase activity, we used Nedd4 as a positive control of E3 activity (Fig. 1A, lane 7). As shown in Fig. 1A, the ubiquitylated proteins were detected in the presence of E1, E2-enzyme mixture, hVPS18, ubiquitin (Fig. 1A, lane 6) but not detected in the absence of either E1, E2, hVPS18, or E. coli lysate (Fig. 1A, lanes 1–5). Therefore, hVPS18 has a ubiquitin ligase activity using bacterial proteins as substrates. In a series of ubiquitylation pathways, it is required for the specific interaction between E3, E2, and substrate, to transfer ubiquitins to a target substrate. To examine the selectivity and the specificity of E2 enzymes for hVPS18, we performed in vitro ubiquitylation assay using various E2 enzymes. As shown in Fig. 1B, hVPS18 has a preference for UbcH4 (Fig. 1B, lane 5). Moreover, we examine whether RING-H2 domain of hVPS18 is responsible for ubiquitin ligase activity. As shown in Fig. 1C, hVPS18ΔRING does not have ubiquitin ligase activity in vitro (Fig. 1C, lane 3), suggesting that VPS18 functions as a ubiquitin ligase depending on its RING-H2 domain.

Identification of SNK That Interacts with hVPS18—To explore the interacting proteins with hVPS18, we screened the rat brain cDNA library by yeast two-hybrid using the full-length human hVPS18 cDNA as a bait. From the screening of 2 × 10^6 clones, we obtained some rat clones encoding the homologues of hVPS11, hVPS16, and hVPS33a (data not shown), that have been shown to constitute a hetero-oligomeric complex with hVPS18, confirming the validity of this screen. Four of the positive clones, two identical clones (clone #A12-1, Fig. 2A) and two independents, have a sequence identity with the rat SNK cDNA (accession number: NM_031821), which was previously characterized as a new type of serine/threonine kinase highly induced in the presence of serum and stimuli that produce synaptic plasticity (14, 15). SNK is one of the Plks that associate with cell cycle (16–18). The full-length SNK cDNA revealed that SNK is a 682-amino acid protein with calculated molecular mass of 77.8 kDa, containing kinase domain at the N-terminal regions and polo-box domain (PBD) at the C-terminal regions (14, 19).

We further confirmed the interaction of hVPS18 and SNK using the two-hybrid assays. Results of β-galactosidase color reaction were summarized in Fig. 2B. The clone A12-1 and full-length SNK bind to both
the full-length hVPS18 (FL) and hVPS18 lacking RING-H2 domain (ΔRING), but not to hVPS18 lacking the C-terminal coiled-coil domain and RING-H2 domain (ΔCC2ΔRING). This suggests that hVPS18 interacts with SNK through the second coiled-coil domain.

**hVPS18 Interacts with SNK in Vitro and in Vivo**—To answer the question whether the full-length SNK interacts with hVPS18, we performed immunoprecipitation assays. HEK293T cells were transfected with Myc-SNK and HA-tagged hVPS11, hVPS18, hVPS16, and hVPS33a, respectively. As shown in Fig. 3A, SNK was efficiently co-immunoprecipitated with hVPS18 and the other Class C VPS. Please note that the interaction between SNK and hVPS18 is strongest among other members of Class C VPS. To further confirm the association of SNK and the Class C VPS in vitro, we performed an in vitro pull-down assay. The bacterial expressed GST-SNK was immobilized on glutathione beads, and insect-expressed His-tagged hVPS11, hVPS18, and hVPS16 were loaded. As shown in Fig. 3B (lanes 1–6), in a good agreement with the data obtained in the yeast two-hybrid screen, GST-SNK preferentially interact with His-hVPS18. As shown in Fig. 3B (lanes 7–12), hVPS11 and hVPS16 were retained with GST-SNK in the presence of hVPS18. These results suggest that the interaction between SNK and other Class C VPS in co-immunoprecipitation assay is partly mediated by hVPS18, and all of the Class C VPS proteins may constitute a large hetero-oligomeric complex in vivo (6).

Because the highly conserved PBD plays an important role for the correct subcellular localization and molecular interaction, we performed in vitro pull-down assays using the bacterially expressed GST proteins fuse to the various deletion forms of SNK. As shown in Fig. 4, SNK full-length and deletion mutants containing PBD at the C-terminal regions, efficiently bind to hVPS18, indicated that the PBD of SNK is involved in protein-protein interaction consistent with previous report (20, 21).

**VPS18 Ubiquitylates SNK in Vitro and in Vivo**—To answer the question whether SNK is ubiquitylated by hVPS18, we performed ubiquitylation assays of SNK in vivo and in vitro. COS7 cells were co-transfected with FLAG-tagged SNK, and various combinations of Myc-tagged hVPS18, hVPS11, and hVPS16, in the presence or absence of HA-tagged ubiquitin. As shown in Fig. 5A, SNK was heavily conjugated with HA-ubiquitin in the cells overexpressing hVPS18 (Fig. 5A, lane 3). The faint smearing in lanes 2, 4, and 5 suggests the possibility that SNK was ubiquitylated by endogenous VPS18 or other anonymous E3 of COS7 cells. It is necessary to observe the direct ubiquitylation of SNK by hVPS18 in vitro, we carried out ubiquitylation assays in which bacterially expressed GST-SNK proteins were incubated in the presence of E1, E2, His-hVPS18, ubiquitin, and ATP. As shown in Fig. 5B, SNK was ubiquitylated dependent on hVPS18, suggesting that SNK is one of the target substrates for hVPS18 E3 ubiquitin ligase.
HeLa Cell Lines That Overexpress hVPS18 or Knockdown hVPS18—
To characterize the physiological relation of SNK and hVPS18, we established HeLa cells that overexpress hVPS18 (HeLa-hVPS18(OE)) or shRNA interference of hVPS18 (HeLa-hVPS18(shRNA)) by lentivirus. After infection with the recombinant lentivirus, HeLa cells were treated with 10 μg/ml Blasticidin S, which is a selection antibiotic drug of the recombinant lentivirus infection. After 7–14 days of incubation with Blasticidin S, the resistant colony was isolated and expanded. To confirm the expression level of hVPS18 and SNK, Western blot analyses were performed. Compared with the control HeLa cell, the cells treated with shRNA interference lentivirus of hVPS18 show up-regulated expression of SNK. Conversely, the cells overexpressing hVPS18 show...
down-regulated SNK (Fig. 6A). To define the subcellular localization of SNK and hVPS18, immunohistochemistry was performed using established HeLa cell lines as shown in Fig. 6B. Up-regulated expression of SNK was observed in the cells that knocked down hVPS18 by shRNA interference lentivirus, whereas down-regulated expression of SNK was observed in the cells overexpressing hVPS18. The reciprocal level of expression between hVPS18 and SNK strongly suggests the endogenous SNK expression is regulated, at least in part, by hVPS18.

To further analyze the role of hVPS18 in the SNK protein turnover, in vivo cell labeling by $[^{35}S]$Met/$[^{35}S]$Cys was performed. As shown in Fig. 7, the three lines of HeLa cells (control, hVPS18(OE), and hVPS18(shRNA)) were labeled and pulse-chased at five time points (0, 15, 30, 60, and 120 min). The total cell lysates were incubated with rat anti-SNK antibody, and the immunoreactive bands were resolved by SDS-PAGE. In general, polyubiquitylated proteins are promptly subjected to degradation by 26 S proteasome. In a good accordance with our results that SNK is ubiquitinylated, the level of SNK protein degraded quickly (half-life, 20 min, Fig. 7B) as shown in the control HeLa cells. When hVPS18 was overexpressed, the degradation of SNK protein was enhanced (half-life, 13.5 min, Fig. 7B). Conversely, the degradation of SNK protein was retarded in the HeLa cells knocked-down hVPS18 expression (half-life, 45 min, Fig. 7B). These results suggest that the degradation of endogenous SNK in HeLa cells can be regulated by the level of hVPS18 expression.

Delayed Entry to S Phase When Overexpressed with hVPS18—Our hypothesis is that hVPS18 is involved in the degradation of SNK. It was previously reported that the kinase activity of SNK is required for centriole duplication close to the G1 to S phase transition (16). Cultured SNK$^- -$ embryonic fibroblasts showed delayed entry to S phase (18). We thus examined the cell-cycle progression of HeLa cells overexpressing hVPS18. Although the profile of cell cycle between control HeLa cells and hVPS18 overexpressing HeLa cells was almost similar, the proportion of cells to S phase after 20 h was significantly lower than the control HeLa cells (Fig. 8). These results are in good accordance with the
null mutant analyses and suggest that the expression level of hVPS18 may regulate the SNK protein level in vivo (18).

DISCUSSION

The main findings of this study are that hVPS18 acts as a genuine ubiquitin ligase to induce the ubiquitylation and degradation of SNK using in vitro and in vivo experiments. The turnover of SNK protein was shortened by the overexpression of hVPS18, meanwhile the turnover was retarded by the shRNA interference of hVPS18. It suggests that the protein level of SNK is regulated by hVPS18. In HeLa cells overexpressing hVPS18, the delayed entry to S phase was observed during the cell-cycle progression. The targeted protein degradation has been increasingly understood to be a general mechanism by which cells properly regulate the intracellular protein levels. In eukaryotic cells, lysosomes/vacuoles are important organelles for the protein degradation. The Class C VPS complex has been intensively characterized as the mole-
cules mediating the vesicle transport between late endosomes and lysosomes (6). Overexpression of VPS18 elicited abnormal perinuclear aggregation of late endosomes and lysosomes (22). Knockdown of VPS18 by RNA interference resulted in dispersion of these organelles throughout the cell periphery (23). Taken together with our current results that hVPS18 functions as ubiquitin ligase, it is possible to propose an overlapped mechanism of protein degradations between endosome/lysosome and ubiquitin-proteasome pathways through hVPS18.

hVPS18 contains several domains that are conserved in multiple species; a putative clathrin heavy chain repeat and coiled-coil domain that may mediate a protein-protein interaction, and C-terminal RING-H2 finger motif (6). The RING-H2 finger is a cysteine-rich, zinc-binding domain that is involved in diverse cellular processes, including transcriptional regulation, RNA transport, signal transduction, and membrane trafficking (9, 11). There are several lines of evidence showing that RING-H2 finger protein may function as a ubiquitin ligase. RING-H2 finger governs the transfer of ubiquitin from E2 to the protein substrate. From our in vitro experiments using anonymous substrates of E. coli, hVPS18 efficiently transferred ubiquitins from UbcH4. Because the recombinant hVPS18 devoid of RING-H2 did not show any E3 activity, the E3 activity of hVPS18 is dependent on its RING-H2 domain. In this report, we identified SNK as one of the target substrates for hVPS18 by the yeast two-hybrid screen. SNK was originally identified as an immediate-early transcript in NIH3T3 cells (14). The deduced amino acid sequences revealed that SNK is a member of Plks that are a family of serine/threonine kinases closely related to polo of Drosophila (24). Plks have been identified in evolutionarily diversified eukaryotes, and they share a conserved N-terminal kinase domain and a homologous non-catalytic C terminus. A domain of 30 amino acid residues is highly conserved among these kinases and thus denoted as the polo-box domain (PBD) (19). One of the functions of PBD was described as a phosphoserine or phosphothreonine binding domain that mediates the targeting of Plks to the subcellular structures such as centrosomes (20, 25, 26).

Previously, it was reported that endogenous SNK expression was regulated by the post-transcriptional level by showing the short half-life of ~15 min (17). Such a short half-life is reasonable from our results that SNK was ubiquitylated and degraded by ubiquitin-proteasome-dependent protein degradation. The Ubiquitylation of SNK by hVPS18 pathway may also play a role in the regulation of protein turnover in response to cellular stress or signaling events. However, the mechanism by which hVPS18 regulates SNK turnover is not fully understood and requires further investigation.
some pathway. But in the overexpression of full-length hVPS18, the SNK degradation is significantly enhanced. Our results suggest that hVPS18 regulates the protein level of SNK through its binding to the poly-domain and ubiquitylation of SNK. Activation of a number of protein kinases, such as protein kinase C (27), Src (28, 29), and ERK1/2 (30), results in ubiquitin/proteasome-mediated degradation that is dependent on their kinase activity. Taken together, SNK is regulated by the two fundamental intracellular mechanisms, phosphorylation and ubiquitin-dependent proteolysis, that control its kinase activity and molecular abundance.

Previous study revealed a functional role of SNK by establishing SNK-null mutant mice (18). Only growth retardation of embryo and slight delay of skeletal development of late gestation were reported as well as a delayed entry to S phase. Such mild phenotypes indicate that members of Piks may mutually compensate their function. Although it has not been clear how much hVPS18 contributes to the physiological protein level of SNK in vivo, the cell-cycle progression is similar between SNK−/− cells and HeLa cells overexpressing hVPS18. We assume that hVPS18 is involved in the degradation of SNK, at least in HeLa cells. In some cases, hVPS18 may not be the only E3 involved in SNK ubiquitylation, because the knock-down of hVPS18 did not completely abolish the ubiquitylation and degradation of SNK in vivo (data not shown). Several different classes of E3 ubiquitin ligases appear to directly regulate the same substrate in the case of p53, Numb, and Notch receptor (31, 32). For the ubiquitylation of Notch receptor, HECT type E3 ubiquitinates the same substrate in the case of p53, Numb, and Notch receptor. Several different classes of E3 ubiquitin ligases appear to directly regulate the same substrate in the case of p53, Numb, and Notch receptor. To identify SNK as a new binding molecule for SPAR (spine-associated Rap homolog), we performed yeast-two hybrid screen to identify proteins that interact with SNK. Recently, Pak and Sheng (35) used the yeast-two hybrid screen to identify SNK as a new binding molecule for SPAR (spine-associated Rap homolog). This screen identified SNK as a new binding molecule for SPAR (spine-associated Rap homolog). Our results suggest that the other pathway(s) for ubiquitylation of SNK may exist. Further analyses will be required to reveal the molecular mechanisms in detail.

Acknowledgments—We greatly appreciate the help and support of Drs. Satoru Massuda, Keiji Wada, Eiki Kominami, Keiji Tanaka, and Yoshishita Kudo. We thank members of the Dept. of Neurochemistry, NCNP, for discussion.

REFERENCES
1. Barr, F. A., Siljé, H. H., and Nigg, E. A. (2004) Nat. Rev. Mol. Cell. Biol. 5, 429–440
2. Moshe, Y., Boulaire, J., Pagano, M., and Herskho, A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7937–7942
3. Casenghi, M., Meraldi, P., Weinhart, U., Duncan, P. L., Korner, R., and Nigg, E. A. (2003) Dev. Cell 5, 113–125
4. Jang, Y. J., Ma, S., Terada, Y., and Erikson, R. L. (2002) J. Biol. Chem. 277, 44115–44120
5. Pak, D. T., and Sheng, M. (2003) Science 302, 1368–1373
6. Kim, B. Y., Kramer, H., Yamamoto, A., Kominami, E., Kohsaka, S., and Akazawa, C. (2001) J. Biol. Chem. 276, 29393–29402
7. Sato, T. K., Rehling, P., Peterson, M. R., and Emr, S. D. (2000) Mol. Cell 6, 661–671
8. Herskho, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
9. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
10. Schnell, J. D., and Hicke, L. (2003) J. Biol. Chem. 278, 35857–35860
11. Freemont, P. S. (2000) Curr. Biol. 10, 884–887
12. Yogosawa, S., Miyazuchi, Y., Honda, R., Tanaka, H., and Yasuda, H. (2003) Biochem. Biophys. Res. Commun. 302, 869 – 872
13. Katayama, K., Wada, K., Miyoshi, H., Ohashi, K., Tachibana, M., Furuki, R., Mizuguchi, H., Hayakawa, T., Nakajima, A., Kadowaki, T., Tsutsui, Y., Nakagawa, S., Kamiyama, T., and Mayumi, T. (2004) FEBS Lett. 566, 178–182
14. Simmons, D. L., Neel, B. G., Stevens, R. E., Dett, G., and Erikson, R. L. (1992) Mol. Cell. Biol. 12, 4164–4169
15. Kaeuselmann, G., Weiler, M., Wulff, P., Albers, S., Konietzko, U., Scalfi, I., Staebli, U., Beretier-Hahn, J., Strebhardt, K., and Dall, H. (1999) EMBO J. 18, 5528–5539
16. Warkne, S., Kemmler, S., Hames, R. S., Tsai, H. L., Hoffmann-Rohrer, U., Fry, A. M., and Hoffmann, I. (2004) Curr. Biol. 14, 1200–1207
17. Ma, S., Liu, M. A., Yuan, Y. L., and Erikson, R. L. (2003) Mol. Cancer Res. 1, 376–384
18. Ma, S., Charmann, J., and Erikson, R. L. (2003) Mol. Cell. Biol. 23, 6936–6943
19. Glover, D. M., Ohta, H., and Tavass, A. (1996) J. Cell Biol. 135, 1681–1684
20. Elia, A. E., Rellores, P., Haier, L. F., Chao, J. W., Iwins, F. J., Hoeppker, K., Mohammad, D., Cantley, L. C., Smerdon, S. J., and Yaffe, M. B. (2003) Cell 115, 83–95
21. Cheng, K. Y., Lowe, E. D., Sinclair, J., Nigg, E. A., and Johnson, L. N. (2003) EMBO J. 22, 5757–5768
22. Sevrioukov, E. A., He, J. P., Mohgrabi, N., Sunnio, A., and Kramer, H. (1999) Mol. Cell 4, 479–486
23. Poupon, V., Stewart, A., Gray, S. R., Piper, R. C., and Luzio, J. P. (2003) Mol. Biol. Cell 14, 4015–4027
24. Hamaanaka, R., Maloid, S., Smith, M. R., O’Connell, C. D., Longo, D. L., and Ferris, D. K. (1994) Cell Growth Differ. 5, 249–257
25. Elia, A. E., Cantley, L. C., and Yaffe, M. B. (2003) Science 299, 1228–1231
26. Jiang, Y. J., Liu, C. Y., Ma, S., and Erikson, R. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1984–1989
27. Lu, Z., Liu, D., Moria, A., Devonish, W., Pagano, M., and Foster, D. A. (1998) Mol. Cell. Biol. 18, 839–845
28. Hakai, Y., and Martin, G. S. (1999) Curr. Biol. 9, 1039–1042
29. Harris, K. F., Shoji, I., Cooper, E. M., Kumar, S., Oda, H., and Howley, P. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13738–13743
30. Lu, Z., Xu, S., Jozzeiro, C., Cob, M. H., and Hunter, T. (2002) Mol. Cell 9, 945–956
31. Nie, J., McGill, M. A., Dermer, M., Dho, S. E., Wolting, C. D., and McClade, G. C. (2002) EMBO J. 21, 93–102
32. Susini, L., Passer, B. J., Amzallag-Elbaz, N., Juven-Gershon, T., Prieur, S., Privat, N., Tynyder, M., Gendron, M. C., Israel, A., Amson, R., Oren, M., and Telerman, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 15067–15072
33. Cornell, M., Evans, D. A., Mann, R., Fostier, M., Flasza, M., Montalbani, M., Artavanis-Tsakonas, S., and Baron, M. (1999) Genetics 152, 567–576
34. Qiu, L., Jozzeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T., and Liu, Y. C. (2000) J. Biol. Chem. 275, 35734–35737
35. Pak, D. T., Yang, S., Rudolph-Correia, S., Kim, E., and Sheng, M. (2001) Neuron 31, 289–303