A Coated Vesicle-associated Kinase of 104 kDa (CVAK104) Induces Lysosomal Degradation of Frizzled 5 (Fzd5) * §

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Receptor internalization is recognized as an important mechanism for controlling numerous cell surface receptors. This event contributes not only to regulate signal transduction but also to adjust the amount of cell surface receptors. Frizzleds (Fzds) are seven-pass transmembrane receptor family proteins for Wnt ligands. Recent studies indicated that Fzd5 is internalized in response to Wnt stimulation to activate downstream signaling pathways. After internalization, it appears that Fzd5 is recycled back to the plasma membrane. However, whether internalized Fzd5 is sorted to lysosomes for protein degradation remains unclear. We here report that a coated vesicle-associated kinase of 104 kDa (CVAK104) selectively induces lysosomal degradation of Fzd5. We identify CVAK104 as a novel binding partner of Dishevelled (Dvl), a scaffold protein in the Wnt signaling pathway. Interestingly, we find that CVAK104 also interacts with Fzd5 but not with Fzd1 or Fzd4. CVAK104 selectively induces intracellular accumulation of Fzd5 via the clathrin-mediated pathway, which is suppressed by coexpression of a dominant negative form of Rab5. Fzd5 is subsequently degraded by a lysosomal pathway. Indeed, knockdown of endogenous CVAK104 by RNA interference results in an increase in the amount of Fzd5. In contrast, Wnt treatment induces Fzd5 internalization but does not stimulate its degradation. Overexpression or knockdown of CVAK104 results in a significant suppression or activation of the Wnt/β-catenin pathway, respectively. These results suggest that CVAK104 regulates the amount of Fzd5 by inducing lysosomal degradation, which probably contributes to the suppression of the Wnt signaling pathway.

Internalization of cell surface receptors is an important event to regulate signal transduction from the extracellular environment (1, 2). This event contributes to control the amount of receptors at the plasma membrane. Internalization mainly occurs via the clathrin-dependent pathway. It is characterized by the recruitment of adaptor protein (AP), 2 such as AP-2, and the assembly of a clathrin coat, which helps the inward budding of clathrin-coated vesicles (3). Internalized receptors are transported to early endosomes, from where they are either recycled back to the plasma membrane or directed to degradative components, such as lysosomes. Rab5, a member of the Rab family GTPase proteins that exert regulatory functions in the endocytic and exocytic trafficking, regulates the fusion of plasma membrane-derived vesicles with early endosomes and homotypic fusion among early endosomes (4).

Accumulating data indicate that numerous regulatory proteins also play important roles in endocytic processes. Coated vesicle-associated kinase of 104 kDa (CVAK104) is one of these accessory proteins, which was recently discovered by mass spectroscopy analysis of AP preparations from bovine brain (5). Several groups reported that CVAK104 interacts with clathrin (5–7). In addition, CVAK104 binds to AP-2 and phosphorylates the β subunit of AP-2 in vitro, suggesting a role in the clathrin-mediated endocytosis (5). Furthermore, it was recently demonstrated that CVAK104 also functions in trafficking between the trans-Golgi network and endosomes. For example, knockdown of CVAK104 by small interfering RNAs (siRNAs) results in mis-sorting of the lysosomal enzyme cathepsin D (6). CVAK104 also regulates sorting of t-SNARE proteins from the trans-Golgi network to late endosomes in which they function as an adaptor for docking and fusion of vesicles (7). These reports suggest an importance of CVAK104 in intracellular trafficking that occurs after endocytosis. The Wnt signaling pathway is evolutionarily conserved from nematodes to mammals and is involved in embryonic development and various human diseases, including cancer (8–10). In this signaling pathway, Dishevelled (Dvl) functions as an essential signal transducer from the Wnt receptors to downstream components. Dvl is composed of three conserved domains: an N-terminal Dishevelled-Axin (DIX) domain, a PSD95/Dlg/ZD1 (PDZ) domain in the middle, and a C-terminal Dishevelled-Egl10-pleckstrin (DEP) domain. It is well known that these three domains are required for protein-protein interaction to transduce signals to downstream targets. Dvl also possesses a region harboring positively charged (basic) amino acid residues (termed the basic region) (11–14). It is reported that the basic region is also required for interaction with several downstream signaling components. Indeed, Frat1 and NRX (nucleoredoxin) interact with Dvl through the basic...
region and the PDZ domain (15, 16). Furthermore, Par1 binds only to the basic region (17). These results suggest that the basic region plays a critical role in the function of Dvl.

Frizzled (Fzd) receptors are seven-pass transmembrane proteins. The Fz genes were first identified in Drosophila in a screen for mutations that disrupt the polarity of epidermal cells in the adult fly (18). Ten genes encoding Fzds have been identified in the human genome (19), and the overall structure of Fzd receptors is well conserved among the 10 proteins and also throughout evolution (20, 21). Accumulating evidence indicates that Fzd receptors are internalized in response to their Wnt ligands. Wnt5a induces the internalization of Fzd4 (22). Wnt3a induces the internalization of Fzd5 via the clathrin-dependent pathway (23). In addition, Wnt11 cooperates with atypical receptor-related tyrosine kinase to promote the internalization of Fzd7 via the β-arrestin-2-dependent pathway (24).

These ligand-dependent internalizations of Fzd receptors are required for activating signaling pathways. Recent studies also demonstrate that Dvl not only functions as a signal transducer but also plays important roles in internalization of the Fzd receptor. It has been reported that Dvl recruits β-arrestin-2 to internalize Fzd4 in response to Wnt5a treatment (22) and that interaction between Dvl and AP-2 is needed to stimulate internalization of Fzd4 (25). After internalization, cell surface receptors are generally recycled back to the plasma membrane or sorted to lysosomes for protein degradation. It has also been reported that Fzd5 internalized in a ligand-dependent manner appears to be recycled back to the plasma membrane, because internalized Fzd5 co-localizes with Rab11, which plays an important role in the recycling process (23). However, whether receptor degradation, another common consequence after receptor internalization, occurs in the case of Fzd5 still remains unknown.

In this study, we search for in vivo Dvl binding partners and identify CVAK104 as a novel Dvl-interacting protein. We also find that CVAK104 interacts with Fzd5 and that expression of CVAK104 induces intracellular accumulation of Fzd5 through the clathrin-dependent pathway. Interestingly, CVAK104 selectively interacts with and induces accumulation of Fzd5 but not Fzd1 or Fzd4. In addition, we find that Fzd5 internalized in the presence of CVAK104 is subsequently degraded by a lysosomal pathway, suggesting a novel mechanism for regulating the turnover of a specific subclass of Fzd receptors.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The cDNAs for full-length human Fzd1 (GenBank™ accession number BC051271), Fzd4 (AY462097), Fzd5 (AB043702), and CVAK104 (BC063798) were cloned by PCR from a 293 cell cDNA library. Point mutation in Fzd5 was introduced with a site-directed mutagenesis kit (Agilent Technologies). Fzd1-CT (amino acids 623–647), Fzd4-CT (amino acids 497–537), and Fzd5-CT (amino acids 517–585) were generated by PCR with appropriate primers. The full-length and deletion constructs of human Dvl1 were prepared in a previous study (16). The deletion constructs of CVAK104, ΔCT1 (amino acids 1–706), ΔCT2 (amino acids 1–386), ΔNT1 (amino acids 387–929), ΔNT2 (amino acids 328–706), and ΔNT3 (amino acids 707–929), were generated by digestion with appropriate restriction enzymes or by PCR with appropriate primers. The mouse Rab5 construct was as previously described (26).

**Cell Culture and Transfection**—L-Wnt3a cells were purchased from the American Type Cell Collection. 293, COS-7, MCF7, and L cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% calf serum. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Cell Lysis, Immunoprecipitation, and Western Blot Analyses**—Cells were harvested with lysis buffer (20 mM Hepes (pH 7.5), 100 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were centrifuged at 15,000 rpm at 4 °C, and the supernatant was incubated with appropriate antibodies and protein G-Sepharose beads (Pierce) for 4 h. Immunoprecipitates were washed four times with lysis buffer, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 10% fat-free dry milk in phosphate-buffered saline with 0.05% Tween 20 and incubated with primary antibodies and then with alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG (Promega).

**Identification of Proteins**—For the identification of Dvl-interacting proteins, lysates of NIH3T3 cells stably expressing GFP, FLAG-Dvl-PDZ/DEP1, or FLAG-Dvl-PDZ/DEP2 were incubated with anti-FLAG M2-agarose beads (Sigma). The beads were washed five times with lysis buffer and suspended in SDS-PAGE sample buffer. The binding proteins were separated by SDS-PAGE and visualized by silver staining. Identification of the binding proteins was performed as described previously (16). In brief, bands of interest were excised from the gels, and the proteins in each gel slice were digested with trypsin. The trypsinized peptides were then extracted from the gels with acetonitrile containing 5% formic acid and 0.1% trifluoroacetic acid. The peptides were subjected to liquid chromatography for purification and were analyzed by matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (4700 Proteomics Analyzer; Applied Biosystems). Detected masses and the sequences of the peptides were subjected to database searches with the Mascot search engine (Matrix Science).

**RNA Interference**—Duplex siRNAs for human CVAK104 and Fzd5 were purchased from Invitrogen. Nucleotide sequences were as follows: CVAK104-siRNA1, sense (5′-UUUAACAG-UAGCAAAGGUGUGGC-3′) and antisense (5′-GCAC-ACACCUGUUGCUACUGUUA-3′); CVAK104-siRNA2, sense (5′-UGUCGAACCGAGUAAUGUGU-GA-3′) and antisense (5′-UCCAACAGUUAACUCGCUUUCGACA-3′); Fzd5-siRNA1, sense (5′-GAGCACCAACCACAUCCCA- UACGAGA-3′) and antisense (5′-UCUCGAGUUGAGUGGUGUGUC-3′); Fzd5-siRNA2, sense (5′-ACUGGUGG- CUCACUGCUACAGUU-3′) and antisense (5′-AAGUAC- UUGAGCAUGACCCAGAUU-3′); and negative control, sense (5′-AAUGGCGCGCUUCUUAAGAGG-3′) and antisense (5′-CCUCUAAAGGACCGACGCAUUU-3′).

**Internalization Assay**—Internalization assay was performed as described previously (23) with a minor modification. In brief,
cells were seeded onto coverslips coated with poly-L-lysine (Sigma) and transfected with Lipofectamine 2000. At 24 or 48 h after transfection, the cells were incubated with internalization medium (Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES/NaOH (pH 7.5), 0.1% bovine serum albumin) for 1 h at 4 °C. After the cells were washed with ice-cold phosphate-buffered saline three times, internalization was initiated by adding warm Dulbecco’s modified Eagle’s medium, and the dishes were transferred to a heated chamber (37 °C, 5% CO₂). To stop endocytosis, the cells were washed three times with ice-cold phosphate-buffered saline. To examine the effects of cholesterol depletion or clathrin inhibitor on the internalization, 293 cells were pretreated with 25 μg/ml nystatin or 100 μM monodansylcadaverine (MDC) for 1 h at 37 °C.

**Immunofluorescence Analysis**—Immunofluorescence analysis was performed as described previously (27). Observation was done with a confocal scanning laser microscope (FLUOVIEW FV1000; Olympus). Fluorescence images were processed with Adobe Photoshop software.

**Antibodies**—The following antibodies were used: mouse monoclonal anti-β-tubulin and monoclonal anti-FLAG M2 antibodies (Sigma); mouse monoclonal anti-EEA1 and monoclonal anti-β-catenin antibodies (BD Biosciences); mouse monoclonal anti-active β-catenin antibody and rabbit polyclonal anti-Fzd5 antibody (Millipore); rabbit polyclonal anti-c-Myc antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit polyclonal anti-GFP antibody (MBL); and Alexa Fluor 488 goat anti-mouse and -rabbit IgGs and Alexa Fluor 546 goat anti-mouse and -rabbit IgGs (Invitrogen). The anti-CVK104 antibody was raised by immunizing rabbits with the C-terminal fragment of human CVAK104 (amino acids 528–929) fused with a His₆ tag and then affinity-purified.

**Reporter Assay**—TCF/LEF-dependent transcription activity was measured as described previously (16) with a minor modification. In brief, 293 cells were transfected with Lipofectamine 2000 and harvested at 48 h after transfection. Reporter activities were measured with a plate reader (LD400; Beckman Coulter) using a dual luciferase reporter assay system (Promega). TCF/LEF activity was defined as the ratio of TOPFLASH/FOPFLASH reporter activities that were normalized on the basis of Renilla luciferase activity. Each assay was performed in duplicate.

**Statistics**—The results are shown as mean ± S.E. of independent multiple assays. p value was determined by Student’s t test or analysis of variance (Fig. 2C).

**Reverse Transcription-PCR Analysis**—Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer’s instructions. cDNA was synthesized from 2 μg of RNA with ReverTra Ace (Toyobo). PCR was performed with the following primer sets: glyceraldehyde-3-phosphate dehydrogenase (5′-GAAAGGTAAGGTCCGAGAC-3′ and 5′-GGAAAGTGGTGGTGG-3′), Fzd1 (5′-GGCAACATAGATTTTCACCTGG-3′ and 5′-CCAGAACCTGATTCGATGTA-3′), Fzd4 (5′-GGTGGATGATCGAGATGAAGAGCT-3′), and Fzd5 (5′-CTTCTTCGATGCGAGATGCTCG-3′ and 5′-CTCATTCTCCTCCTTTTACCGG-3′).

**RESULTS**

**Identification of CVAK104 as a Novel Dvl-interacting Protein**—To explore novel proteins that interact with the basic region of Dvl, we generated NIH3T3 murine fibroblast-derived cell lines stably expressing FLAG-tagged Dvl deletion mutants, Dvl-PDZ/DEP1 or Dvl-PDZ/DEP2 (Fig. 1A). Dvl-PDZ/DEP1 contains the basic region and PDZ and DEP domains, whereas Dvl-PDZ/DEP2 lacks only the basic region. Therefore, we could expect to find binding partners for the basic region, by comparing co-precipitated proteins with each Dvl deletion mutant. Immunoprecipitation with anti-FLAG antibodies and following silver staining revealed several co-precipitated proteins (Fig. 1B). Among them, we analyzed the ~100 kDa molecular mass protein (indicated with the upper arrowhead in Fig. 1B), which was specifically coimmunoprecipitated with FLAG-Dvl-PDZ/DEP1, but not with FLAG-Dvl-PDZ/DEP2. By mass spectrometry, we identified it to be CVAK104, which was originally identified as a kinase that phosphorylates β2-subunit of AP-2 (5). NRX, which was previously reported as a binding protein to the basic PDZ region in Dvl (16), was also identified (indicated with the lower arrowhead in Fig. 1B). An antibody against CVAK104 was raised in rabbits (supplemental Fig. S1) and used for an immunoprecipitation analysis to confirm endogenous complex formation between Dvl and CVAK104. A positive signal of Dvl was identified in anti-CVK104 precipitates but not in precipitates with control rabbit IgG (Fig. 1C), indicating their in vivo complex formation.

We next investigated the region of Dvl involved in the interaction with CVAK104. We expressed Myc-tagged CVAK104 and FLAG-tagged Dvl deletion constructs in COS-7 cells, and the cell lysates were immunoprecipitated with anti-FLAG antibody. Among Dvl deletion mutants, DvlΔDI1 and PDZ/DEP1 interacted with Myc-CVK104, suggesting that the basic region of Dvl is required for interaction with CVAK104 (Fig. 1D). We next constructed CVAK104 deletion mutants (Fig. 1E) and performed immunoprecipitations to test their abilities to interact with Dvl. As shown in Fig. 1F, both the N-terminal kinase-like domain (ΔCT2) and the C-terminal region (ΔNT3) associated with Dvl, suggesting that CVAK104 harbors two regions that can independently bind to Dvl. We also examined direct interaction between Dvl and CVAK104 using purified proteins. Pull-down assays showed an obvious direct interaction of recombinant His-CVK104 with glutathione S-transferase-Dvl-His (supplementary Fig. S2), confirming that Dvl binds to CVAK104 directly.

**CVAK104 Selectively Induces Intracellular Accumulation of Fzd5**—Recent studies revealed that Dvl plays important roles in internalization of Fzd receptors (22, 25). In addition, CVAK104 has been suggested to be involved in the clathrin-dependent endocytosis (5) and intracellular protein trafficking (6, 7). These results suggested a possibility that CVAK104 plays a role in the turnover of Fzd receptors from the plasma membrane. We generated a series of expression constructs for Fzd receptors (Fzd1, Fzd4, and Fzd5) fused with green fluorescence protein (GFP) at their C termini and investigated the internalization of Fzds in 293 cells. When 293 cells expressing Fzd-GFP were subjected to an internalization assay, we found that each
Fzd-GFP localized predominantly at the cell surface (Fig. 2A). We next cotransfected 293 cells with each Fzd-GFP and FLAG-CVAK104. We observed a GFP signal in a dotlike pattern in Fzd5-GFP-transfected cells but not in Fzd1- or Fzd4-GFP-transfected cells (Fig. 2B). However, only part of the intracellular Fzd5-GFP dots overlapped with FLAG-CVAK104, suggesting that Fzd5 and CVAK104 do not constitutively form a protein complex. Approximately 70% of cells expressing Fzd5-GFP with FLAG-CVAK104 showed such vesicular accumulation of Fzd5-GFP, whereas Fzd1- or Fzd4-GFP-expressing cells were not affected by coexpression of FLAG-CVAK104 (Fig. 2C).

To examine at which intracellular compartment Fzd5-GFP accumulated, we performed immunostaining with antibodies for various localization marker proteins. We found that Fzd5-GFP dots induced by expression of CVAK104 partially co-localized with EEA1 (Fig. 3A), a marker for the early endosomes, implying that intracellular accumulation of Fzd5 might be caused by internalization via the conventional endocytic pathway. Therefore, we next examined whether this Fzd5 accumulation was caused through either the clathrin- or caveolin-mediated pathway. We tested the effect of two different inhibitors, which block either the clathrin- or caveolin-dependent endocytosis. MDC inhibits the formation of the clathrin-coated pit and, thus, has been widely used to inhibit the clathrin-dependent endocytosis (28). In the presence of MDC, CVAK104-induced accumulation of Fzd5...
Lysosomal Degradation of Fzd5 by CVAK104

We next investigated whether Dvl affected the interaction between CVAK104 and Fzd5. We transfected 293 cells with plasmids encoding FLAG-CVAK104, Fzd5-GFP, and Myc-Dvl and performed immunoprecipitations with an anti-FLAG antibody. Interestingly, coexpression of Myc-Dvl clearly blocked the interaction between FLAG-CVAK104 and Fzd5-GFP, whereas Myc-Dvl-ΔDIX2, which did not interact with CVAK104 (Fig. 1D), did not interfere with their interaction (Fig. 4C). To further examine the interaction among these three proteins, we constructed a mutant of Fzd5 in which the Lys residue in the Lys-Thr-X-X-Trp motif was converted to Ala residue (K525A). The Lys-Thr-X-X-Trp motif is conserved in all human Fzd receptors and required for the interaction with Dvl (30). Consistent with a previous report (30), Fzd5 K525A had little if any ability to interact with Dvl (data not shown). We tested whether the K525A conversion in Fzd5 affected the interaction with CVAK104. However, we did not observe any significant differences when we used the K525A mutant form of Fzd5, instead of wild type Fzd5 (Fig. 4D). Therefore, Dvl inhibits the interaction between Fzd5 and CVAK104, presumably through its direct interaction with CVAK104.

As shown above, ectopically expressed Dvl disturbed the interaction between CVAK104 and Fzd5 (Fig. 4, C and D). We next treated transfected 293 cells with Wnt3a-conditioned medium (CM) and performed immunoprecipitation analyses. We found that the interaction between CVAK104 and Fzd5 was interrupted in response to Wnt3a CM treatment (Fig. 4E). We also tested whether Wnt stimulation affected the interaction between CVAK104 and Dvl. In contrast to the case of Fzd5, the affinity of CVAK104 to Dvl was increased in response to treatment with Wnt3a CM (Fig. 4F). These results suggest that activation of Wnt signaling negatively regulates the interaction between CVAK104 and Fzd5, probably through Dvl.

Fzd5 Is Degraded by a Lysosomal Pathway—Internalized cell surface receptors are thought to be directed to two distinct destinations: to lysosomes for degradation or to the cell surface. It is reported that Fzd5 internalized in response to Wnt stimulation appears to be recycled back to the plasma membrane (23). We found that Wnt signaling negatively regulates the

1E) revealed that Fzd5 associated with the N-terminal kinase-like domain and the C-terminal region of CVAK104 (Fig. 4B). Interestingly, these regions overlap with the Dvl-binding regions (Fig. 1F), suggesting the possibility that Dvl and Fzd5 compete with each other for binding to CVAK104.

FIGURE 3. CVAK104-induced Fzd5 accumulation occurs via the clathrin-dependent pathway. A, 293 cells were transfected with Fzd5-GFP and FLAG-CVAK104. At 48 h after transfection, cells were subjected to an internalization assay and visualized by direct observation of GFP fluorescence and staining with anti-EEA1 antibody. A merged image of the signals for GFP (green) and EEA1 (red) is also indicated. Scale bar, 5 μm. B, 293 cells expressing Fzd5-GFP and FLAG-CVAK104 were pretreated with DMSO, MDC, or nystatin for 1 h and were then subjected to an internalization assay in the presence of inhibitors. Scale bar, 5 μm. C, quantification of cells with internalized Fzd5-GFP in B. All data are mean ± S.E. (n = 3), *p < 0.01. D, 293 cells expressing Fzd5-GFP and FLAG-CVAK104 with or without Myc-Rab5 S34N were subjected to an internalization assay and then visualized by direct observation of GFP fluorescence and immunostained with anti-FLAG antibody. Scale bar, 5 μm.

was obviously blocked (Fig. 3, B and C). However, nystatin, a blocker of the caveolae/raft-dependent endocytosis (29), did not affect it (Fig. 3, B and C). We also examined the effect of Rab5, which is a key regulatory molecule directing vesicle transport and endosomal fusion in the early endocytic pathway (4), on CVAK104-induced accumulation of Fzd5. Coexpression with a dominant negative form of Rab5 (Rab5 S34N) was found to efficiently suppress accumulation of Fzd5 induced by CVAK104 expression (Fig. 3D). Collectively, the intracellular accumulation of Fzd5 induced by expression of CVAK104 appears to be mediated by the clathrin- and Rab5-dependent endocytosis.

CVAK104 Selectively Interacts with Fzd5—Since ectopic expression of CVAK104 induces selective accumulation of Fzd5 (Fig. 2), we addressed whether CVAK104 interacts with Fzd5. We expressed Fzd-GFP alone or with FLAG-CVAK104 in 293 cells and then performed immunoprecipitation analyses. We found that among Fzd receptors, only Fzd5-GFP co-precipitated with FLAG-CVAK104 (Fig. 4A). Pull-down assays using purified proteins demonstrated an interaction between His-CVAK104 and glutathione S-transferase-tagged cytoplasmic tail of Fzd5 but not of Fzd1 or Fzd6 (supplemental Fig. S3), which indicates that CVAK104 directly and selectively interacts with Fzd5. We further investigated the region of CVAK104 involved in interaction with Fzd5. Overexpression and immunoprecipitation analyses with CVAK104 deletion mutants (Fig. with Dvl (30).
interaction between CVAK104 and Fzd5 (Fig. 4, C and E), and there is a report that CVAK104 is involved in protein sorting to lysosomes (6). Therefore, we speculated that Fzd5 internalized in the presence of CVAK104 is sorted to lysosomes rather than recycled back to the plasma membrane. To elucidate the lysosomal degradation of Fzd5, we treated 293 cells with the lysosomotropic drug chloroquine, which increases endosomal and lysosomal pH and impairs lysosomal function (31, 32). Chloroquine treatment resulted in marked intracellular accumulation of Fzd5-GFP, although cells were neither treated with Wnt3A CM nor transfected with CVAK104 (Fig. 5A). We could not observe any significant intracellular accumulation of Fzd5-GFP by blocking proteasomal activity with MG132 (Fig. 5A). We noticed a significant decrease of Fzd5-GFP at the plasma membrane by chloroquine treatment. This reduction would be caused by some indirect or nonspecific effect of chloroquine, because it has been reported that chloroquine also interferes with endosomal sorting and recycling (33).

We further tested whether Fzd5 was sorted to lysosomes for protein degradation by biochemical analyses. We generated lines of 293 cells that stably express GFP-tagged Fzd1, Fzd4, and Fzd5 (293 Fzd-GFP) to examine the amount of Fzd receptors. We transfected 293 Fzd-GFP cells with mock or FLAG-CVAK104-encoding plasmids and then performed immunoblotting analyses. Immunoblotting with anti-GFP antibody showed that the amount of Fzd5-GFP was decreased by expression of CVAK104, whereas other Fzd receptors were not (Fig. 5B), which clearly correlated with the selective accumulation of Fzd5 (Fig. 2, B and C). Next, we investigated the effect of Wnt3a CM on Fzd5 degradation. We treated 293 Fzd5-GFP cells with Wnt3a CM and performed immunoblotting. In contrast to CVAK104 expression, treatment with Wnt3a did not affect the amount of Fzd5-GFP (Fig. 5C). We also examined the effect of inhibitors for lysosomal or proteasomal degradation. Treatment of 293 Fzd5-GFP cells with chloroquine resulted in an increase in the amount of Fzd5-GFP, whereas treatment with MG132 showed no significant changes in the amount of Fzd5-GFP, compared with control treatment (Fig. 5D). Furthermore, treatment with chloroquine rescued the expression level of Fzd5-GFP reduced by expression of CVAK104 (Fig. 5D). Collectively, Fzd5 that is internalized in the presence of CVAK104 is presumably sorted to lysosomes for degradation.

To further confirm the lysosomal degradation of Fzd5, we performed loss-of-function analyses of endogenous CVAK104. We employed two different siRNAs, CVAK104-siRNA1 and -2, to reduce expression of CVAK104. We treated 293 Fzd5-GFP cells with Wnt3a CM and performed immunoblotting. In contrast to CVAK104 expression, treatment with Wnt3a did not affect the amount of Fzd5-GFP (Fig. 5F). We also examined the effect of inhibitors for lysosomal or proteasomal degradation. Treatment of 293 Fzd5-GFP cells with chloroquine resulted in an increase in the amount of Fzd5-GFP, whereas treatment with MG132 showed no significant changes in the amount of Fzd5-GFP, compared with control treatment (Fig. 5D). Furthermore, treatment with chloroquine rescued the expression level of Fzd5-GFP reduced by expression of CVAK104 (Fig. 5D). Collectively, Fzd5 that is internalized in the presence of CVAK104 is presumably sorted to lysosomes for degradation.
Therefore, CVAK104 appears to negatively regulate the Wnt/β-catenin pathway compared with control siRNA treatment (Fig. 6). A modest but significant increase of the reporter fluorescence resulted in a modest but significant increase of the reporter fluorescence. Scale bar/H9252 (anti-active E-cadherin antibodies). After transfection, the lysates were subjected to immunoblotting with the indicated antibodies.

FIGURE 5. Lysosomal degradation of Fzd5. A, 293 cells expressing Fzd5-GFP were treated with DMSO, chloroquine (100 μM), or MG132 (20 μM) for 10 h. The cells were fixed and visualized by direct observation of GFP fluorescence. Scale bar/H9252 (anti-active E-cadherin antibodies). B, 293 cells stably expressing Fzd5-GFP were transfected with an empty (-) or FLAG-CVAK104 expression vector (+), and then cell lysates were analyzed by immunoblotting with the indicated antibodies. C, 293 cells stably expressing Fzd5-GFP were transfected with an empty (-) or FLAG-CVAK104 expression vector (+) or treated with control (-) or Wnt3a CM (+). The lysates were subjected to immunoblotting analysis with the indicated antibodies. D, 293 cells stably expressing Fzd5-GFP were transfected with an empty (-) or FLAG-CVAK104 expression (+) vector. Cells were treated with 100 μM chloroquine (Chlo) or 20 μM MG132 (MG) for 10 h. The lysates were subjected to immunoblotting with the indicated antibodies. E, 293 cells stably expressing Fzd5-GFP were transfected with control or CVAK104-siRNAs. At 48 h after transfection, the lysates were subjected to immunoblotting with the indicated antibodies.

the involvement of endogenous CVAK104 in the regulation of the amount of Fzd5.

CVAK104 Negatively Regulates the Wnt/β-Catenin Pathway—We finally examined the importance of CVAK104 in the Wnt/β-catenin pathway using 293 cells. We first confirmed the endogenous expression of Fzd5 mRNA and protein in 293 cells (supplemental Fig. S4). We transfected 293 cells with FLAG-CVAK104 and performed reporter assays for the Wnt/β-catenin pathway. Overexpression of FLAG-CVAK104 suppressed the reporter activity induced by treatment with Wnt3a CM (Fig. 6A). In contrast, treatment with siRNAs for CVAK104 resulted in a modest but significant increase of the reporter activity compared with control siRNA treatment (Fig. 6B). Therefore, CVAK104 appears to negatively regulate the Wnt/β-catenin pathway. To confirm this finding, we next investigated the activation status of β-catenin, using the antibody (anti-active β-catenin) that specifically recognizes the dephosphorylated form of β-catenin. Treatment of 293 cells with Wnt3a CM resulted in an increase of the signal, which was suppressed by expression of FLAG-CVAK104 (Fig. 6C). In addition, knockdown of endogenous CVAK104 caused a slight increase in the amount of activated β-catenin (Fig. 6D). Collectively, these results implicate CVAK104 in the regulation of the Wnt signaling pathway.

DISCUSSION

Accumulating data indicate that several Fzd receptors are internalized in a ligand-dependent manner (22–24). In regard to Fzd5, it is internalized in response to Wnt3a stimulation (23). After internalization, it appears that Fzd5 is recycled back to the plasma membrane. In this study, we demonstrated that CVAK104 selectively interacted with Fzd5 and induced lysosomal degradation. The fate of Fzd5 internalized in the presence of CVAK104 is clearly different from that in response to Wnt3a, indicating a novel regulatory aspect of Fzd receptors.

It is reported that CVAK104 localizes at the peripheral vesicular structures that are accessible to endocytosed cell surface receptors, in addition to its predominant localization in the perinuclear region (6). Therefore, internalized Fzd5 might be trapped in endosomal compartments through the interaction with CVAK104, which was subsequently sorted to the lysosomal pathway (Fig. 5). Alternatively, CVAK104 might directly induce internalization of Fzd5, because one study suggested that CVAK104 is involved in the clathrin-dependent endocytosis (5). However, another study indicated that CVAK104, either endogenous or exogenously expressed, was not found at the plasma membrane (6). Therefore, further studies are needed to clarify the mechanism of Fzd5 accumulation induced by CVAK104.

Importantly, we found that CVAK104 selectively induced the intracellular accumulation of Fzd5 but not of Fzd1 or Fzd4 (Fig. 2, B and C). This receptor selectivity of CVAK104 should be explained by the specific interaction between CVAK104 and Fzd5 (Fig. 4, A and B). Fzd family proteins are composed of an N-terminal extracellular region containing a cysteine-rich domain that is required for the interaction with Wnt ligands, a seven-pass transmembrane segment, and a C-terminal cytoplasmic tail. The sequences in the cysteine-rich domain and the intracellular loops between the transmembrane segments among Fzd receptors are very similar to each other. However, the C-terminal cytoplasmic tails of Fzd receptors differ significantly (34), which might explain the specific interaction between CVAK104 and Fzd5.
Our data demonstrated that Dvl functions as a negative regulator of the interaction between CVAK104 and Fzd5. We found that Dvl blocked the CVAK104-Fzd5 interaction by binding to CVAK104 (Fig. 4, C and D). We also found that treatment with Wnt CM resulted in down-regulation of the CVAK104-Fzd5 interaction (Fig. 4E). Dvl has been reported to accumulate to the membrane in response to Wnt stimulation (35–37). Therefore, we consider that membrane-recruited endogenous Dvl may disturb the CVAK104-Fzd5 complex formation. In fact, Wnt treatment enhanced the interaction between CVAK104 and Dvl (Fig. 4F). These results suggest an interesting possibility that activation of Wnt signaling would suppress the CVAK104-dependent lysosomal sorting of Fzd5. On the basis of this idea, treatment with Wnt ligands should cause an increase of Fzd receptor. However, we did not observe so significant change in the amount of Fzd5-GFP irrespective of Wnt stimulation (Fig. 5C). Piddini et al. (38) reported that Drosophila Fzd2 is presumably internalized by the interaction with its ligand Wingless and then transported to the lysosomes, suggesting the existence of Wnt-induced degradation mechanism of Fzd. Therefore, the level of Fzd proteins appears to be regulated in a complex manner by multiple mechanisms, which should be the focus for future studies.

One important issue to be addressed is how Fzd5 is sorted to lysosomes by CVAK104. Several recent studies have shown the importance of phosphorylation in the lysosomal degradation of cell surface receptors. For example, it is reported that phosphorylation of the cytoplasmic tail of PAR1 (protease-activated receptor-1), a GPCR for thrombin, is important in both internalization and degradation (39, 40). Furthermore, it is also demonstrated that the α-factor pheromone receptor is phosphorylated and subsequently ubiquitinated to be internalized and degraded (41). These reports raise an interesting possibility that the kinase activity of CVAK104 might play an important role in lysosomal sorting of Fzd5. CVAK104 belongs to the SCY1-like family of protein kinases (referred to as SCY1L2) that are, however, generally thought to be catalytically inactive (42). Conner and Schmid (5) demonstrated that, in the presence of poly-l-lysine, recombinant CVAK104 proteins expressed in insect cells phosphorylate the β2 subunit of AP-2 in vitro (5). Düwel and Ungewickell (6), however, could not confirm the kinase activity of CVAK104 (6). Therefore, whether CVAK104 functions as a kinase remains a controversial issue to be clarified in the future.

Recent studies demonstrated the presence of several proteins that regulate the postendocytic sorting of GPCRs to lysosomes. It is reported that sorting nexin 1 regulates lysosomal targeting of PAR1 (43) and that GPCR-associated sorting protein 1 interacts with a specific subclass of opioid receptors and dopamine receptors, targeting them to lysosomes for protein degradation (44, 45). Together with the similarity of Fzds to GPCRs (46), we can expect the presence of some lysosomal sorting mechanism specific to Fzd5, in which CVAK104 plays an important role.

The effect of overexpression or knockdown of CVAK104 on the Wnt/β-catenin pathway was moderate (Fig. 6). However, it should be noted that similar moderate effects have been reported in several studies. For example, knockdown of NRX also resulted in a similar level of increase (about 2-fold) of the TCF reporter activity (16). In addition, overexpression of Idax suppresses the reporter activity in response to Wnt3a treatment (from ~2.2- to 1.2-fold) (47) at a similar intensity of CVAK104. Developmental analyses using Xenopus clearly confirmed the physiological importance of both nucleoredoxin and Idax in the Wnt/β-catenin pathway (16, 48). It should also be noted that CVAK104 regulates the Wnt/β-catenin pathway via the specific subclass of Fzd receptors, such as Fzd5. Thus, it is rather reasonable that the effect is only partial, not complete, in 293 cells. CVAK104 probably regulates the signaling in some special developmental context in which Fzd5 plays a dominant role as a Wnt receptor.

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REFERENCES

1. Di Fiore, P. P., and De Camilli, P. (2001) Cell 106, 1–4
2. Conner, S. D., and Schmid, S. (2003) Nature 422, 37–44
3. Traub, L. M. (2005) Biochim. Biophys. Acta 1744, 415–437
4. Soldati, T., and Schliwa, M. (2006) Nat. Rev. Mol. Cell Biol. 7, 897–908
5. Conner, S. D., and Schmid, S. L. (2005) J. Biol. Chem. 280, 21539–21544
6. Düwel, M., and Ungewickell, E. J. (2006) Mol. Cell. Biol. 17, 4513–4525
7. Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Lee, J. J., Tilghman, S. M., Gumbiner, B. M., and Costantini, F. (1997) Cell 90, 181–192
8. Sun, T. Q., Lu, B., Feng, J. J., Reinhard, C., Jan, Y. N., Fantl, W. J., and Williams, L. T. (2001) Cell Biol. 3, 628–636
9. Hicke, L., Zanolari, B., and Riezman, H. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6546–6551
10. Chen, W., ten Berge, D., Brown, J., Ahn, S., Hu, L. A., Miller, W. E., Caron, M. G., Barak, L. S., Nusse, R., and Lefkowitz, R. J. (2003) Science 301, 1391–1394
11. Yamamoto, H., Komekado, H., and Kikuchi, A. (2006) Dev. Cell 11, 213–223
12. Kim, G. H., Her, J. H., and Han, J. K. (2008) J. Cell Biol. 182, 1073–1082
13. Yu, A., Rual, J. F., Tamai, K., Harada, Y., Vidal, M., He, X., and Kirsch-hassen, T. (2007) Dev. Cell 12, 129–141
14. Kuroda, T. S., Fukuda, M., Ariga, H., and Mikoshiba, K. (2002) J. Biol. Chem. 277, 9212–9218
15. Terabayashi, T., Funato, Y., and Miki, H. (2003) Biochem. Biophys. Res. Commun. 375, 660–665
16. Schütze, S., Machleidt, T., Adam, D., Schwandner, R., Wiegmann, K., Kruse, M. L., Heinrich, M., Wickel, M., and Krönke, M. (1999) J. Biol. Chem. 274, 10203–10212
17. Rothberg, K. G., Heuser, J. E., Donzelli, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992) Cell 68, 673–682
18. Cong, F., Schweizer, L., and Varmus, H. (2004) Development 131, 5103–5115
19. Mellman, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700
20. Dean, R. T., Jessup, W., and Roberts, C. R. (1984) Biochem. J. 217, 27–40
21. Gramp, T., Sauter, K., Markovic, B., and Benke, D. (2007) J. Biol. Chem. 282, 24157–24165
22. Wang, H. Y., Liu, T., and Malbon, C. C. (2006) Cell Signal. 18, 934–941
23. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) Science 298, 1912–1934
24. Keller, R. (2002) Science 298, 1950–1954
25. Terabayashi, T., Itoh, T. J., Yamaguchi, H., Yoshimura, Y., Funato, Y., Ohno, S., and Miki, H. (2007) J. Neurosci. 27, 13098–13107
26. Piddini, E., Marshall, F., Dubois, L., Hirst, E., and Vincent, J. P. (2005) Development 132, 5479–5489
27. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 32874–32880
28. Paing, M., Stutts, A. B., Kobout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) J. Biol. Chem. 277, 1292–1300
29. Hicke, L., Zanolari, B., and Riezman, H. (1998) J. Cell Biol. 141, 349–358
30. Rothbächer, U., Laurent, M. N., Dear dorff, M. A., Klein, P. S., Cho, K. W., and Fraser, S. E. (2000) EMBO J. 19, 1010–1022
31. Gullapalli, A., Wolfe, B. L., Griffin, C. T., Magnuson, T., and Trejo, J. (2006) Mol. Biol. Cell 17, 1228–1238
32. Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., Murray, S. R., and Von Zastrow, M. (2002) Science 297, 615–620
33. Bartlett, S. E., Enquist, J., Hopf, F. W., Lee, J. H., Gladher, F., Kharazia, V., Waldo hoer, M., Mailliard, W. S., Armstrong, R., Bonci, A., and Whistler, J. L. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 11521–11526
34. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) Annu. Rev. Biochem. 67, 653–692
35. Hino, S., Kishida, S., Michiu e, T., Fukui, A., Sakamoto, I., Takada, S., Asahima, M., and Kikuchi, A. (2001) Mol. Cell Biol. 21, 330–342
36. Michiue, T., Fukui, A., Yukita, A., Sakurai, K., Danno, H., Kikuchi, A., and Asahima, M. (2004) Dev. Dyn. 230, 79–90
37. Terabayashi, T., Itoh, T. J., Yamaguchi, H., Yoshimura, Y., Funato, Y., Ohno, S., and Miki, H. (2007) J. Neurosci. 27, 13098–13107
38. Piddini, E., Marshall, F., Dubois, L., Hirst, E., and Vincent, J. P. (2005) Development 132, 5479–5489
39. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 32874–32880
40. Paing, M., Stutts, A. B., Kobout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) J. Biol. Chem. 277, 1292–1300
41. Hicke, L., Zanolari, B., and Riezman, H. (1998) J. Cell Biol. 141, 349–358
42. Rothbächer, U., Laurent, M. N., Dear dorff, M. A., Klein, P. S., Cho, K. W., and Fraser, S. E. (2000) EMBO J. 19, 1010–1022
43. Gullapalli, A., Wolfe, B. L., Griffin, C. T., Magnuson, T., and Trejo, J. (2006) Mol. Biol. Cell 17, 1228–1238
44. Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., Murray, S. R., and Von Zastrow, M. (2002) Science 297, 615–620
45. Bartlett, S. E., Enquist, J., Hopf, F. W., Lee, J. H., Gladher, F., Kharazia, V., Waldo hoer, M., Mailliard, W. S., Armstrong, R., Bonci, A., and Whistler, J. L. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 11521–11526
46. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) Annu. Rev. Biochem. 67, 653–692
47. Hino, S., Kishida, S., Michiu e, T., Fukui, A., Sakamoto, I., Takada, S., Asahima, M., and Kikuchi, A. (2001) Mol. Cell Biol. 21, 330–342
48. Michiue, T., Fukui, A., Yukita, A., Sakurai, K., Danno, H., Kikuchi, A., and Asahima, M. (2004) Dev. Dyn. 230, 79–90