ArPIKfyve Regulates Sac3 Protein Abundance and Turnover

**DISRUPTION OF THE MECHANISM BY Sac3I41T MUTATION CAUSING CHARCOT-MARIE-TOOTH 4J DISORDER**

The mammalian phosphatidylinositol (3,5)-bisphosphate (PtdIns(3,5)P₂) phosphatase Sac3 and ArPIKfyve, the associated regulator of the PtdIns3P-5 kinase PIKfyve, form a stable binary complex that associates with PIKfyve in a ternary complex to increase PtdIns(3,5)P₂ production. Whether the ArPIKfyve-Sac3 subcomplex functions outside the PIKfyve context is unknown. Here we show that stable or transient expression of ArPIKfyveWT in mammalian cells elevates steady-state protein levels and the PtdIns(3,5)P₂-hydrolyzing activity of Sac3, whereas knockdown of ArPIKfyve has the opposite effect. These manipulations do not alter the Sac3 mRNA levels, suggesting that ArPIKfyve might control Sac3 protein degradation. Inhibition of protein synthesis in COS cells by cycloheximide reveals remarkably rapid turnover of expressed Sac3WT ($t_{1/2} = 18.8$ min), resulting from a proteasome-dependent clearance as evidenced by the extended Sac3WT half-life upon inhibiting proteasome activity. Coexpression of ArPIKfyveWT, but not the N- or C-terminal halves, prolongs the Sac3WT half-life consistent with enhanced Sac3 protein stability through association with full-length ArPIKfyve. We further demonstrate that mutant Sac3, harboring the pathogenic Ile-to-Thr substitution at position 41 found in patients with CMT4J disorder, is similar to Sac3WT with regard to PtdIns(3,5)P₂-hydrolyzing activity, association with ArPIKfyve, or rapid proteasome-dependent clearance. Remarkably, however, neither is the steady-state Sac3I41T elevated nor is the Sac3I41T half-life extended by coexpressed ArPIKfyveWT, indicating that unlike with Sac3WT, ArPIKfyve fails to prevent Sac3I41T rapid loss. Together, our data indentify a novel regulatory mechanism whereby ArPIKfyve enhancesSac3 abundance by attenuating Sac3 proteasome-dependent degradation and suggest that a failure of this mechanism could be the primary molecular defect in the pathogenesis of CMT4J.

The phosphatase Sac3 and the kinase PIKfyve² are responsible for synthesis and turnover of phosphatidylinositol (PtdIns) (3,5)P₂ in mammalian cells (1). Intriguingly, both the endogenous and the ectopically expressed enzymes are found to reside in the same regulatory complex, called the PAS complex, or PIKfyve-ArPIKfyve-Sac3, organized by the PIKfyve regulator ArPIKfyve and its ability to homooligomerize (2, 3). Despite their antagonistic activities, PIKfyve and Sac3 appear to be enzymatically active in the ternary complex. Thus, in the case of PIKfyve, formation of the PAS regulatory core is critical for PIKfyve activation (3). Likewise, the Sac3 phosphatase retains its PtdIns(3,5)P₂-hydrolyzing activity within the PAS ternary complex (4). These data reveal an unusual paradigm whereby a common complex relays two opposing activities, one for synthesis, another for degradation, the physiological meaning of which is yet to be understood (1, 5). Data from in vitro reconstitution studies indicate increased and decreased PtdIns(3,5)P₂ levels triggering mammalian endosome fission and fusion, respectively (2, 6). Thus, an association of the two active yet antagonistic enzymes in a common complex would be consistent with the critical requirement for a tight control of PtdIns(3,5)P₂ homeostasis related to dynamic endosome membrane remodeling through fission and fusion (1). Understanding the spatial and temporal regulation and the coordination of the individual enzyme activities within the PAS complex is essential in providing a better comprehension of the intricate PtdIns(3,5)P₂ homeostatic mechanism. Maintaining PtdIns(3,5)P₂ homeostasis is apparently indispensable for life as evidenced by the early lethality of Drosophila melanogaster or Caenorhabditis elegans PIKfyve-null mutants (7, 8) and by the early death of ArPIKfyve and Sac3-deficient mouse models (9, 10). Concordantly, a defective Sac3 I41T allele in combination with a null allele is responsible for the pathogenesis of Charcot-Marie-Tooth type 4J (CMT4J) peripheral neuropathy, a recessively inherited disease with early onset manifested by progressive motor and sensory neuron degeneration (10, 11). The molecular and cellular mechanisms rendering this single I41T amino acid substitution pathogenic are currently unknown.

ArPIKfyve associates with the Sac3 phosphatase independently of PIKfyve in a stable ArPIKfyve-Sac3 heterooligomer (3, 4). This binary association is apparently a prerequisite for a productive ternary PAS complex formation and PIKfyve activation, but whether it influences functionality of the Sac3 phosphatase and/or the ArPIKfyve scaffold is unknown. In the present study, we have investigated the role of ArPIKfyve for Sac3 regulation in both native and ectopically transfected cells. We report here that ArPIKfyve markedly elevates Sac3 protein levels through a mechanism that involves a delay of rapid Sac3 turnover by the proteasome pathway. We further reveal that unlike with

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² The abbreviations used are: PIKfyve, phosphoinositide kinase for position five containing a fyve finger domain; ArPIKfyve, associated regulator of PIKfyve; PI, phosphoinositide; PAS, PIKfyve-ArPIKfyve-Sac3; Sac3, Sac domain-containing phosphatase 3; PtdIns, phosphatidylinositol; eGFP, enhanced green fluorescence protein; HA, hemagglutinin; qRT-PCR, quantitative RT-PCR; CMT4J, Charcot-Marie-Tooth type 4J.
Sac3WT, ectopically expressed ArPIKfyve fails to prevent the loss of Sac3I41T, the mutant form with the pathogenic Ile-to-Thr substitution found in patients with the CMT4J disorder. These results identify a novel regulatory mechanism involving an ArPIKfyve-dependent prevention of Sac3 degradation and suggest that a failure in this mechanism could be the primary molecular defect underlying the pathogenesis of CMT4J.

EXPERIMENTAL PROCEDURES

Antibodies, siRNAs, and cDNA Constructs—Polyclonal anti-ArPIKfyve (R7069), anti-Sac3, anti-ArPIKfyve (WS047), or anti-HA (R4289) antibodies were used for immunoprecipitation and Western blotting as detailed previously (12). Monoclonal anti-α-tubulin antibody and anti-Myc producing 9E10.2 hybridoma cells were from Sigma and ATCC, respectively. Polyclonal anti-GFP (Ab290) and anti-actin antibodies were from Abcam and Sigma, respectively. SMARTpool® siRNA duplexes (Dharmacon) targeting human and mouse sequences of ArPIKfyve (M-015729; M-040510), PIKfyve (M-005058; M-040127), or control cyclophilin B (d-001136) were characterized elsewhere (3, 13, 14). pEGFP-HA-ArPIKfyveWT, pEF-BO5-Myc-Sac3WT, pEGFP-Sac3−WT, and pCMV5-Myc- or pEGFP-HA-ArPIKfyve constructs were generated previously (2, 4). Generation of the pEGFP-Sac3I41T construct is detailed in the supplemental Experimental Procedures.

Cell Cultures, Treatments, and Transfections—Differentiation of 3T3L1 adipocytes and maintenance of COS7 and HEK293 cells were described previously (4, 13). Generation of a HEK293 cell line stably expressing HA-ArPIKfyveWT is detailed in the supplemental Experimental Procedures. Cell transfection with siRNAs and analyses 72 h after transfection were detailed previously (3, 13). cDNA-transfected COS7 cells were treated 24 h after transfection with cycloheximide (0.2 mg/ml), MG132 (10μM), and/or lactacystin (10μM) for the time periods indicated in the figure legends.

32P Cell Labeling and Detection of Radiolabeled PIs—The stable HEK293-HA-ArPIKfyveWT cell line, induced or not induced with doxycycline (1μg/ml), was labeled in phosphate/serum-free DMEM for 2.5 h at 37°C with [32P]orthophosphate as described previously (2, 14). Extracted radiolabeled lipids were deacylated and analyzed by HPLC (2, 14).

[35S]Methionine Cell Labeling—Differentiated 3T3L1 adipocytes were labeled in methionine/serum-free DMEM with 300μCi/ml [35S]methionine/cysteine for 12 h at 37°C as detailed previously (15). Cells were then chased for the time intervals indicated in the figure legends and processed for immunoprecipitation and immunoblotting as detailed elsewhere (15).

Isolation of RNA and Quantitative RT-PCR—RNA isolation from 3T3L1 adipocytes was by the TRizol reagent protocol. The qRT-PCR was performed according to published protocols (16) detailed in the supplemental Experimental Procedures.

Immunoprecipitation, Immunoblotting, and In Vitro Phosphatase Assay—These assays were conducted exactly as described previously (2).

Other Assays, Quantitation, and Statistics—Protein concentration was determined by bicinchoninic acid protein assay. Protein levels were quantified from the intensity of the immunoblot bands by a laser scanner (Microtek) and UN-SCAN-IT software (Silk Scientific). Data evaluation by exponential or linear regression analyses was performed by Microsoft Excel. Data are presented as means ± S.E. Statistical analysis was performed by Student’s t test, with p < 0.05 considered as significant.

RESULTS AND DISCUSSION

We have observed previously (2) and confirmed herein a significant reduction in endogenous Sac3 protein levels upon ArPIKfyve knockdown in mammalian cells (Fig. 1). Several well-defined criteria (17) were used to qualify this off-target effect as specific. Thus, it was manifested by different siRNA pools targeting mouse and human ArPIKfyve sequences in mouse 3T3L1 adipocytes and human HEK293 cells, respectively (Fig. 1, A and B). Next, reduced Sac3 levels were documented under different approaches for siRNA cell delivery such as cationic lipids or electroporation, under which conditions PIKfyve, the kinase that associates with the Sac3-ArPIKfyve subcomplex, remained insignificantly changed (Fig. 1, A and B). Finally, unlike with ArPIKfyve, siRNA-mediated depletion of PIKfyve in both cell types failed to significantly alter the Sac3 protein level (Fig. 1C, and not shown). By contrast, siRNA-mediated Sac3 depletion in different mammalian cell types was without a specific off-target effect on either ArPIKfyve or PIKfyve levels (2, 12). To determine whether the reduction in Sac3 protein levels is due to decreased transcription under these conditions, transcript levels were analyzed by quantitative RT-PCR. Although ArPIKfyve mRNA was reduced by ~88%, levels of Sac3 mRNA remained unaltered (Fig. 1D). These data indicate that the loss of ArPIKfyve down-regulates the Sac3 protein levels through a mechanism that involves reduced translation and/or increased degradation.

To confirm the above observation by the reverse approach, we generated a stable HEK293 cell line inducibly expressing HA-ArPIKfyve under tetracycline-controlled reverse transactivation. Screening by immunoblotting identified a cell clone, clone 23, in which HA-ArPIKfyveWT was expressed at a level ~2-fold higher versus endogenous ArPIKfyve, as measured 20 h after induction with doxycycline (supplemental Fig. 1A). Importantly, under induced ArPIKfyveWT expression, we found endogenous Sac3 protein to also be elevated (supplemental Fig. 1A), although to a lesser extent (2 ± 0.2-fold). By contrast, endogenous levels of PIKfyve remained unchanged, as were levels of several unrelated proteins (supplemental Fig. 1A). Importantly, the elevation in the Sac3 protein under induced ArPIKfyve expression corresponded to the increased Sac3 phosphatase activity as revealed by HPLC analyses of [32P]Pis extracted from the [35S]-labeled clone 23 HEK293 cell line (supplemental Fig. 1B). Quantitation of HPLC profiles from four independent cell-labeling experiments documented a 45% increase in the PtdIns3P/PtdIns(3,5)P2 ratio resulting from both an increase in PtdIns3P and a decrease in PtdIns(3,5)P2 levels (supplemental Fig. 1B). No such changes were documented upon doxycycline induction of the control cell line (data not shown). Together, these data indicate that induced expression of ArPIKfyveWT increases protein levels of active Sac3 phosphatase, capable of hydrolyzing PtdIns(3,5)P2 to PtdIns3P.
To mechanistically characterize the role of ArPIKfyve in regulating Sac3 protein levels, we sought to reproduce this effect with ectopically expressed proteins in a transiently transfected cell system. Examination of lysates from singly or doubly transfected COS cells revealed a profound (~3-fold) elevation of steady-state Sac3WT upon coexpression of ArPIKfyveWT versus single Sac3WT expression (Fig. 2A). An ArPIKfyveWT-dependent increase in Sac3WT protein accumulation was seen with different tags (HA, Myc, and eGFP on either one of the two proteins) and was highly specific as evidenced by the inability of coexpressed PIKfyveWT or other coexpressed proteins, such as JLP or GD1-1, to increase steady-state protein levels of Sac3WT (Fig. 2, A, B, and D, and not shown). Considering increased Sac3 protein stability within the Sac3-ArPIKfyve heterooligomer as a potential mechanism for elevating the Sac3WT protein in the presence of ArPIKfyveWT, we next examined whether the ArPIKfyve C-terminal fragment (residues 523–732) that binds Sac3WT as effectively as ArPIKfyveWT could reproduce this effect. Intriguingly, however, unlike ArPIKfyveWT, coexpression of eGFP-ArPIKfyveC3 to a level similar to that of eGFP-ArPIKfyveWT did not increase steady-state Sac3WT as evidenced by the similar band intensities of coexpressed and singly expressed Myc-Sac3WT (Fig. 2A). Likewise, the eGFP-ArPIKfyve N-terminal fragment (residues 1–511) that does not bind Sac3WT to a substantial extent (4) did not bring about an accumulation in Myc-Sac3WT steady-state levels (Fig. 2A). Thus, although the ArPIKfyve C-terminal region harbors the major Sac3-binding determinants, it is insufficient to mediate the steady-state accumulation of Sac3WT protein. Rather, this effect requires the entire ArPIKfyve protein molecule.

To determine whether rapid proteolysis may explain the markedly reduced steady-state Sac3WT levels in the absence of coexpressed ArPIKfyveWT, COS cells transiently transfected with Sac3WT alone or together with ArPIKfyveWT were treated for various times with the protein synthesis inhibitor cycloheximide. The time course for Sac3WT disappearance was monitored by immunoblotting. In the absence of protein synthesis, we observed a remarkably rapid decline of eGFP-Sac3WT levels with time, indicating a fast eGFP-Sac3WT turnerover rate (Fig. 2B). From the exponential equation fitted to the data of seven time course experiments, where the band intensity of individual time points was normalized to the 0-min time point, we calculated the half-life for singly expressed Sac3WT to be only 18.8 min (Fig. 3C). Coexpression of ArPIKfyveWT not only increased steady-state Sac3WT levels but profoundly slowed the rate of Sac3WT turnover evidenced by extended half-life calculated from the plotted data (t1/2 = 49 min; Fig. 2, B and C). In contrast, under cycloheximide-inhibited protein synthesis, immunoreactive levels of eGFP-ArPIKfyveWT and eGFP-PIKfyveWT expressed separately or together, remained practically unaltered throughout the period of investigation, further reinforcing the specificity of ArPIKfyveWT in regulating Sac3 turnover rate (Fig. 2D). These data also indicate that unlike Sac3WT, expressed ArPIKfyveWT or PIKfyveWT exhibits remarkable protein stability with very long half-lives. Corroborating the observations for slow turnover of ectopically expressed ArPIKfyveWT and PIKfyveWT, the endogenous ArPIKfyve and PIKfyve proteins were also found to display long half-lives (t1/2 = 33 h for PIKfyve and 32 h for ArPIKfyve), as found by the pulse-chase experiments in 3T3L1 adipocytes metabolically labeled with [35S]methionine (supplemental Fig. 2, A and B). It should be noted, however, that the half-life of endogenous Sac3 was not possible to be accurately measured under similar pulse-chase experiments in 3T3L1 adipocytes due to a very weak [35S] band comigrating with immunoprecipitated endogenous Sac3 as a result of insufficient [35S]methionine incorporation likely associated with the fast turnover rate relative to the chase time (data not shown). Notwithstanding this observation, the documented rapid proteolysis and profoundly extended half-life of ectopically expressed Sac3WT in the absence and presence of coexpressed ArPIKfyveWT, respectively, are consistent with the notion that the ArPIKfyve-Sac3 heterodimer, Sac3 protein is stabilized and protected from rapid degradation.

Rapid protein turnover is typically associated with degradation by the proteasome pathway (18, 19). To test whether Sac3WT follows this degradation itinerary, we used two well-characterized 26 S proteasome inhibitors, lactacystin and the aldehyde peptide MG132 (20), and treated eGFP-Sac3WT-expressing COS cells for various times. Intriguingly, both lactacystin and MG132 induced an accumulation of Sac3WT in a time-dependent manner, consistent with Sac3WT degradation by the proteasome (Fig. 2E). Likewise, when new synthesis of eGFP-Sac3WT protein was blocked with cycloheximide for 3 h, the fast disappearance of Sac3WT was slowed in the presence of proteasome inhibition (Fig. 2F). Together, these data are consistent with the notion that in the absence of ArPIKfyveWT, ectopically expressed Sac3WT undergoes a rapid degradation by the 26 S proteasome.

The Sac3 phosphatase is encoded by a single-copy gene positioned on chromosome 6q21 in humans (1). Mutations in the human gene were recently found to be responsible for the recessively inherited disorder CMT type 4J, a severe form of Charcot-Marie-Tooth disease with early onset, affecting sensory and motor neurons (10, 11). The CMT4J patients are compound heterozygotes carrying one loss-of-function allele and a missense I141T mutation in the second allele. To reveal the molecular mechanism whereby 141T mutation deteriorates Sac3 functionality in these...
patients, we first tested plausible alterations in the in vitro phosphatase activity of the Sac3$^{I41T}$ mutant. Anti-eGFP immunoprecipitates derived from transfected COS cells expressing eGFP-Sac3$^{I41T}$ or eGFP-Sac3$^{WT}$ to comparable levels (Fig. 3B) were analyzed by the malachite green assay, measuring the amounts of released inorganic phosphate due to Sac3 phosphatase activity (2, 12). We observed no significant differences in the ability of the two Sac3 proteins to hydrolyze di-C8-PtdIns(3,5)P2 (Fig. 312). We observed no significant differences in the ability of the two Sac3 proteins to hydrolyze di-C8-PtdIns(3,5)P2 (Fig. 3). This result is also corroborated by the fact that the I41T mutation is positioned outside the phosphatase domain (residues 154−547; Ref. 2), making alterations in the enzymatic activity unlikely. Likewise, lack of significant differences between the ability of eGFP-Sac3$^{WT}$ and eGFP-Sac3$^{I41T}$ to associate in a binary complex with ectopically expressed ArPIKfyve$^{WT}$ or in a ternary complex with ArPIKfyve$^{WT}$ and PIKfyve$^{WT}$ was documented by immunoprecipitation assays in transfected COS cells (Fig. 3B and data not shown). The nearly intact association displayed by the Sac3$^{I41T}$ mutant is in accordance with the ArPIKfyve-binding region in Sac3, mapped at the C-terminal half in both yeast and mammalian Sac3 (4, 21).

We next tested whether Sac3$^{I41T}$ differs from Sac3$^{WT}$ in the herein documented rapid Sac3$^{WT}$ turnover rate and the ability of coexpressed ArPIKfyve to delay this fast protein clearance. To this end, COS cells ectopically expressing Sac3$^{I41T}$ alone or together with ArPIKfyve$^{WT}$ were treated for various times with cycloheximide. In the absence of ArPIKfyve, Sac3 remains unfolded and, hence, prone to rapid destruction. A critical role in this regulatory mechanism is attributed to the Ile-to-Thr mutation at position 41 in Sac3, which determines the ability of ArPIKfyve to protect the Sac3 molecule from degradation. Because the I41T substitution does not significantly affect the ArPIKfyve-Sac3 association (Fig. 3) and lies outside the

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**FIGURE 2.** ArPIKfyve$^{WT}$ increases steady-state levels, extends the half-life, and delays proteasome degradation of ectopically expressed Sac3$^{WT}$. COS cells were singly or doubly transfected with the cDNA constructs as indicated. Twenty-four h after transfection, cells were treated with cycloheximide (0.2 mg/ml; B–D and F), lactacystin (10 μM; E), and/or MG132 (10 μM; E and F) for the denoted time periods. Equal protein amounts of cell lysates were analyzed by immunoblotting with the indicated antibodies (A, B, and D–F). Illustrated are chemiluminescence detections of representative blots out of 2–7 independent experiments for each condition. C, Sac3$^{WT}$ degradation rate in the absence or presence of ArPIKfyve$^{WT}$, shown in B, is averaged from 3–7 independent experiments for each time point, normalized to the corresponding 0-min point, and fitted by exponential curve.
Sac3 major contact sites, as determined by coimmunoprecipitation by immunoblotting with the indicated antibodies (A) or analyzed directly by immunoblotting subsequent to cell lysates were immunoprecipitated (B) with anti-GFP (C) or with the indicated antibodies (D). D, Sac3I41T degradation rate in the absence or presence of ArPIKfyveWT, shown in C, is averaged from 3 independent experiments for each time point, normalized to the corresponding 0-min point, and fitted by exponential curve.

Sac3 major contact sites, as determined by coimmunoprecipitation in mammalian cells (4) or yeast two-hybrid interactions with the yeast Sac3 counterpart (21), the mode whereby the Sac3I41T escapes the ArPIKfyve-dependent stabilization is currently unclear. According to recent homology structural modeling of the Sac phosphatase domain, the I41T substitution may affect the Sac3 major contact sites, as determined by coimmunoprecipitation by immunoblotting with the indicated antibodies (B). D, Sac3I41T degradation rate in the absence or presence of ArPIKfyveWT, shown in C, is averaged from 3 independent experiments for each time point, normalized to the corresponding 0-min point, and fitted by exponential curve.

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