Inhibitory effect of SPE-39 due to tyrosine phosphorylation and ubiquitination on the function of Vps33B in the EGF-stimulated cells

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Abstract

Although SPE-39 is a binding protein to Vps33B that is one of the subunit in the mammalian HOPS complex, the elements of SPE-39 function remain unknown. Here, we show that tyrosine phosphorylation of SPE-39 following EGF stimulation plays a role in the stability of SPE-39 itself. Ubiquitination of the C-terminal region of SPE-39 was also elevated in response to EGF stimulation, and this process was regulated by the phosphorylation of Tyr-11 in SPE-39. However, association of Vps33B with SPE-39 inhibited the elevation of ubiquitination of SPE-39 following EGF stimulation, which might be responsible for the stabilization of SPE-39. Furthermore, an opposing functional relationship between SPE-39 and Vps33B on the downregulation of the EGF receptor was observed in EGF-stimulated COS-7 cells.

1. Introduction

The homotypic fusion and vacuole protein sorting (HOPS) complex originally discovered in yeast, has essential roles in fusion of membranes with lysosomes or lysosome-related organelles in metazoans [1,3]. The HOPS complex comprises with four subunits of class C vacuolar protein sorting (Vps) proteins (Vps11, Vps16, Vps18 and Vps33) and two class B Vps proteins (Vps39 and Vps41) [2–4].

Recently, mutations in the gene encoding human Vps33B were reported to arthrogryposis-renal dysfunction-cholestasis syndrome (ARC), which is associated with platelet dysfunction and abnormalities in polarized liver and kidney cells [5].

SPE-39 has been identified as a protein that binds to Vps33 in Caenorhabditis elegans; this interaction is essential for vesicular trafficking during spermatogenesis [6]. The human ortholog of SPE-39 (hSPE-39) is also known to associate with both subtypes of Vps33A and Vps33B in mammalian cells [4,7]. Furthermore, other research groups found hSPE-39 mutations, but not Vps33B mutations in individuals with ARC. The complex formed between hSPE-39, which is also named VIPAR (Vps33B-interacting protein involved in polarity and apical protein restriction), and Vps33B might be involved in the stabilization of the apical membrane proteins [8]. However, the precise mechanisms by which this occurs remain obscure.

SPE-39 (also known as FLJ12707 or C14ORF133) was originally identified through tyrosine phosphoproteomics, and it has been reported that Tyr-11 is phosphorylated following certain stimuli in cultured cells [9–11]. However, further characteristics of its tyrosine phosphorylation have yet to be conducted.

In this study, we confirmed the phosphorylation of Tyr-11 in SPE-39 upon EGF stimulation. SPE-39 is an ubiquitinated protein, and the level of its ubiquitination is elevated in EGF-stimulated cells. We focused on the stability and function of SPE-39 in response to EGF stimulation, and the relationship between SPE-39 tyrosine phosphorylation and ubiquitination and the SPE-39/Vps33B complex.
2. Materials and methods

2.1. Cell culture, transfection, and reagents

COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 units/ml of penicillin. Transfection was carried out by electroporation using the Gene-Pulser system (Bio-Rad). EGF (100 ng/ml, Sigma) dissolved in serum-free medium was added.

2.2. cDNA cloning and vector constructions

SPE-39 (FLJ12707) cDNA, provided by the National Institute of Technology and Evaluation, Japan, was subcloned into pFLAG-CMV6a (Sigma) to be expressed as an N-terminal FLAG-tagged. Vps33B cDNAs were purchased from Open Biosystems, inserted into CMV6a (Sigma) to be expressed as an N-terminal FLAG-tagged.

2.3. Antibodies

An anti-SPE-39 rabbit polyclonal antibody was raised against peptides having an additional cysteine residue at the N-terminal end of the SPE-39 amino acid sequence, KGDEEEYWNSSKF (amino acids 5–17). The peptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits. The antibody was purified using HiTrap N-hydroxysuccinimide-activated Sepharose columns (GE Healthcare) coupled with an immunizing antigen. An anti-EGF receptor rabbit polyclonal antibody was raised against a 15-amino acid peptide corresponding to the C terminus of the protein [12]. Other antibodies were obtained commercially from various companies: anti-FLAG M2 (Sigma), anti-phosphotyrosine (4G10; Upstate Biotech Inc.), anti-Vps33B (Abcam), and anti-HA antibody (12CA5; Roche).

2.4. Immunoprecipitation and immunoblot analysis

The following procedures were carried out at temperatures ranging from 0 to 4 °C. The transfected cells were lysed in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, and a complete protease inhibitor mixture (Roche Applied Science) to produce a total cell lysate (TCL). For the immunoprecipitation experiments, the total cell lysate was centrifuged, and the supernatant was incubated for 2 h with either the primary antibody or an anti-FLAG affinity gel (Sigma). Protein G-Sepharose (GE Healthcare) was added, and the resulting mixture was rotated at 4 °C for 1 h. The beads were subsequently washed 3 times with the lysis buffer. The processed samples were treated as described previously.

2.5. Fluorescence microscopy analysis

Transfected cells were fixed and observed as described previously [13].

3. Results

3.1. Identification of the tyrosine phosphorylation site in SPE-39 upon EGF stimulation

Although SPE-39 protein functions as a Vps33B-interacting protein involved in polarity and apical protein restriction, it was originally identified as a tyrosine-phosphorylated protein in phosphoproteomic studies. Tyr-11 was identified as the site of phosphorylation in fibroblast growth factor-3 (FGF3)-stimulated cells, as well as in pervanadate- and calyculin-treated T-cell leukemia cells [9–11].

We determined that SPE-39 is also phosphorylated upon EGF stimulation of COS-7 cells, and Tyr-11 was observed to be a major site of phosphorylation (Fig. 1B, C). The surrounding amino acid sequence of Tyr-427 is also a consensus motif for phosphorylation by the EGF receptor (Fig. 1A). However, immunoprecipitation studies using deletion mutants revealed that EGF treatment did not result in Tyr-427 phosphorylation (Fig. 1B).

We also determined the effect of EGF stimulation on the interactions between endogenous SPE-39 and Vps33B in HeLa cells. Compared to non-stimulated cells, the amount of Vps33B bound to SPE-39 was slightly reduced in EGF-stimulated cells (Fig. 1D). Next, COS-7 cells expressing FLAG–SPE-39 and GFP–Vps33B were lysed, and the FLAG–SPE-39 protein was immunoprecipitated with the anti-FLAG antibody. Again, the amount of GFP–Vps33B bound to the immunoprecipitated FLAG–SPE-39 was slightly decreased in EGF-stimulated cells compared to non-stimulated cells. This decrease was not observed with the Y11F mutant of SPE-39 (Fig. 1E). Thus, tyrosine phosphorylation of SPE-39 upon EGF stimulation has an inhibitory effect on the binding of SPE-39 and Vps33B.

3.2. Effect of SPE-39 tyrosine phosphorylation and ubiquitination on the interaction between SPE-39 and Vps33B

SPE-39 has been identified as a protein that interacts with Vps33, the latter being a component of the HOPS complex. This complex has important roles for distribution of ubiquitinated cargo protein from the plasma membrane to lysosomes. Therefore, we speculated that this protein complex plays some roles in the ubiquitin–proteasome pathway.

Anti-FLAG immunoprecipitation experiment was performed with lysate from cells expressing FLAG-tagged SPE-39 and HA-tagged ubiquitin. In COS-7 cells, several bands conjugated with HA-tagged ubiquitin might represent polyubiquitinated SPE-39 (Fig. 2B, lane 2). Interestingly, the level of ubiquitination of a C-terminal truncated mutant of SPE-39 (1–400) was decreased compared with the wild type or the mutant of SPE39 (1–420) (Fig. 2B, lane 4 and 6). Therefore, the ubiquitination site in SPE-39 might be located in its C-terminal region.

Ubiquitination of some proteins is upregulated in cells with growth factors stimulation [14,15]. The ubiquitination of SPE-39 was upregulated by EGF-stimulation (Fig. 2C, lane 3). However, this elevation did not occur in cells overexpressing GFP–Vps33B (Fig. 2C, lanes 4 and 5). We also found that the level of poly-ubiquitination of SPE-39, but not of the SPE-39(Y11F) tyrosine phosphorylation mutant, increased with EGF stimulation (Fig. 2C, lanes 6 and 7). Therefore, tyrosine phosphorylation of SPE-39 may regulate its EGF-mediated ubiquitination.


Multiple posttranslational modifications occur in SPE-39 in an EGF-dependent manner, and we found that exogenously expressed FLAG–SPE-39 was degraded in cells that had been treated with EGF for several hours (Fig. 3A, set of left 3 lanes). However, this instability of SPE-39 was limited by the overexpression of GFP–Vps33B (Fig. 3A, set of middle 3 lanes). Furthermore, upon EGF treatment, the degradation of the SPE-39(Y11F) phosphorylation mutant in the absence of GFP–Vps33B was attenuated compared with that of the wild-type protein (Fig. 3A, set of right 3 lanes). Therefore, the instability of SPE-39 was suppressed in the absence of its phosphorylation.
Next, we focused on the functional relationship between SPE-39 and the EGF receptor. GFP–Vps33B was mainly co-localized with the EGF receptor in the COS-7 cells stimulated with EGF for 30 min (Fig. 3C, d–f). In addition, expression of GFP–Vps33B accelerated the downregulation of the EGF receptor in the EGF-stimulated COS-7 cells (Fig. 3B, middle panel). In contrast, exogenous expression of SPE-39 inhibited the co-localization of endogenous EGF receptor and GFP–Vps33B. Co-immunoprecipitation studies were carried out using cell lysates from HeLa cells (D) or the COS-7 cells overexpressing FLAG–SPE-39 (E). Endogenous SPE-39 and each FLAG-tagged variant were immunoprecipitated with an anti-SPE39 and an anti-FLAG antibody, respectively. Immunoblot analysis was carried out using indicated antibodies. Bands were quantitated using reverse image scanning densitometry by normalizing the band intensity of the co-immunoprecipitated Vps33B to the band intensity of the immunoprecipitated endogenous SPE-39 (D) or FLAG–SPE-39 (E). The representative results from three independent experiments are indicated below each panel. The amounts of co-immunoprecipitated Vps33B from non-stimulated cells are normalized to the control. FL: FLAG-tagged, Y11F: SPE-39 mutant with Tyr-11 replaced by Phe.

4. Discussion

Each mutation of SPE-39 or Vps33 is linked to disordered apical protein restriction in the liver and kidney in ARC [5,7]. This is in part because the levels of E-cadherin at the adherens junctions and the level of CEACAM5 at the apical membrane protein are regulated by Vps33B and SPE-39. Reducing the expression of each protein by knockdown or knockout induced degradation of the apical membrane protein, because of its mis-sorting to basolateral membranes and into the late endosomes and lysosomes [7]. For proper maintenance of this pathway, the expression levels of each protein must be correctly regulated. However, the precise mechanisms by which these proteins regulated are unknown. In this study, we provide experimental evidence that post-translational modification of SPE-39 has a significant role in its stability. Specifically, the phosphorylation of Tyr-11 and ubiquitination near the C-terminus of SPE-39 might be closely linked in EGF-stimulated cells. We showed that SPE-39 was protected against proteolytic degradation by association with Vps33B and the interaction between SPE-39 and Vps33B was weakly inhibited by EGF stimulation (Fig. 1D, E). Therefore, each modification of SPE-39—its association with Vps33B and phosphorylation of its Tyr-11 residue—might have opposing effects on its stabilization in the EGF-stimulated cells. Furthermore, Tyr-11 phosphorylation of SPE-39 might regulate ubiquitination of SPE-39 in response to EGF stimulation.
(Fig. 2B). The physiological function of the SPE-39/Vps33B complex is to ensure that the normal structure of the liver and kidney cells, with apical basolateral polarity, is maintained by regulating the expression of adherent proteins such as E-cadherin. EGF and HGF are important factors regulating the formation of a liver progenitor cell line [16]. Cell motility is upregulated in cells with growth
factor stimulation, and this upregulation might inhibit cell–cell contact during cell growth or differentiation. It is possible that the degradation of SPE-39 induced by its secondary modifications is involved in controlling the strength of cell–cell contacts due to reduced expression of adherent proteins.

The EGF receptor is internalized from the plasma membrane into the lysosomes upon ligand stimulation [17,18], and several HOPS-related proteins can affect the distribution and degradation of the EGF receptor. For example, degradation of the EGF receptor is dramatically reduced by depletion of Vps11 [19]. Depletion of SPE-39 induced a delay in the downregulation of the EGF receptor 30 min after the addition of EGF. However, after several hours of EGF treatment, the amount of the EGF receptor was almost identical in cells with or without depletion of SPE-39 [4].

In this study, we confirmed the enhancement of the EGF receptor degradation by overexpression of Vps33B (Fig. 3B). In cells with low-level expression of SPE-39, the HOPS complex including GFP–Vps33B might be involved in the EGF-positive vesicles, which accelerates the deliver of the EGF receptor to the lysosomes. However, response to EGF stimulation and subcellular localization of the HOPS complex may change or be disturbed by the high-affinity interaction of Vps33B and SPE-39 in cells overexpressing...

Fig. 3. (A) Effect of Vps33B expression on the protein stability of SPE-39 in response to EGF stimulation. The amount of each protein was monitored by immunoblotting with specific antibodies. Proteins (20 µg) were applied from each total cell lysate prepared from COS-7 cells expressing each protein stimulated with EGF for the indicated times. The levels of the FLAG–SPE-39 expression were quantified by densitometry. The representative results from three independent experiments are indicated below each panel. The amounts of FLAG–protein at each time point are normalized to the control (lane of each 0 time). (B) Vps33B accelerated the downregulation of the EGF receptor, which was suppressed by co-expression of SPE-39. Effects of GFP–Vps33B and FLAG–SPE-39 expression on the downregulation of the EGF receptor. The amount of each protein was monitored by immunoblotting with anti-EGF receptor antibody. Twenty micrograms of protein of each whole cell lysate prepared from COS-7 cells transfected with the mock vector (upper panel), GFP–Vps33B (middle panel), or both GFP–Vps33B and SPE-39 stimulated with EGF for the indicated times was applied. The amounts of EGF receptor expression were quantified by densitometry. The representative results from three independent experiments are indicated below each panel. The amounts of EGF receptor at each time point are normalized to the control (lane of each 30 min). (C) Subcellular localization of FLAG–SPE-39, EGF receptor and GFP–Vps33B with EGF treatment. COS-7 cells expressing FLAG–SPE-39 (a–c), GFP–Vps33B (d–f) or both proteins (g–j) with EGF treatment for 30 min were processed for immunofluorescence staining using anti-FLAG (a and h) and anti-EGF receptor (b, e and i), respectively. The scale bars represent 10 µm.
FLAG–SPE-39 (Fig. 3C). Because the stability and the quantity of SPE-39 in cells were altered with its phosphorylation and ubiquitination, these modifications may affect the function of the HOPS complex.

The mechanism of endocytic trafficking is conserved from yeast to humans, and the factors in each species that participate in these events are also highly conserved [1,2]. However, several subtypes of the ESCRT and HOPS complexes have been identified in humans but not in yeasts [2,20]. Furthermore, posttranslational modifications are diverse among each subtype [21]. Thus, further studies to characterize the relationship between the tyrosine phosphorylation of SPE-39 and its stability in EGF-stimulated cells are warranted.

References


