pp60c-Src Phosphorylates and Activates Vacuolar Protein Sorting 34 to Mediate Cellular Transformation

Dianne S. Hirsch, Yi Shen, Milos Dokmanovic, and Wen Jin Wu

Abstract
Vacuolar protein sorting 34 (VPS34) contributes to the regulation of the mammalian target of rapamycin complex 1/S6 kinase 1 pathway downstream of nutrient signaling. However, intracellular mechanisms leading to VPS34 activation remain unclear. Here, we report that Src directly phosphorylates VPS34, and that this phosphorylation activates VPS34 lipid kinase activity, leading to Src-Y527F-mediated cellular transformation. Silencing endogenous VPS34 specifically inhibits Src-Y527F–induced colony formation in soft agar, but not Ras-G12V–induced colony formation. We have identified two novel hVPS34 mutations, which either eliminate lipid kinase activity (kinase-dead mutant) or reduce tyrosine phosphorylation by Src-Y527F. When kinase-dead mutant of hVPS34 is stably expressed in Src-Y527F–transformed cells, transformation activities are blocked, indicating that the lipid kinase activity of hVPS34 is essential for Src-mediated cellular transformation. Furthermore, stable expression of this hVPS34 kinase-dead mutant causes an increased number of binucleate and multinucleate cells, suggesting that the kinase activity of hVPS34 is also required for cytokinesis. Moreover, when the hVPS34 mutant that has reduced tyrosine phosphorylation by Src is stably expressed in Src-Y527F–transformed cells, Src-Y527F–stimulated colony formation is also reduced. Data presented here provide important evidence that VPS34 lipid kinase activity could be positively regulated by Src-mediated tyrosine phosphorylation in mammalian cells. This finding highlights a previously unappreciated relationship between VPS34, a class III phosphatidylinositol-3-kinase, and Src non–receptor tyrosine kinase. Additionally, we find that the levels of VPS34 expression and tyrosine phosphorylation are correlated with the tumorigenic activity of human breast cancer cells, indicating that Src to VPS34 signaling warrants further investigation as a pathway contributing to the development and progression of human cancers.

Introduction
Phosphatidylinositol-3-kinases (PI3K) phosphorylate phosphatidylinositol and its phosphorylated derivatives at the 3 position of the inositol ring to generate second messengers that control cell proliferation, survival, motility, membrane trafficking, and morphology (1). PI3Ks are classified into three different classes by substrate specificity and subunit organization (2). Vacuolar protein sorting 34 (VPS34) is a class III PI3K, which only catalyzes phosphorylation on phosphatidylinositol (2, 3). Human VPS34 (hVPS34) is an important component in the regulation of vesicular trafficking, autophagy, and heterotrimeric G protein signaling (4–8). Earlier studies have shown that growth factors and hormones regulate mammalian target of rapamycin (mTOR)/S6 kinase 1 (S6K1) activation via class I PI3K. Recent studies indicate that hVPS34 regulates the nutrient input into the mTOR complex 1/S6K1 signaling pathway (9–12). These results have led to the hypothesis that S6K1 activation might be mediated by both class I PI3K and VPS34 through parallel pathways (10, 11).

In regulating proliferation and tumorigenesis, it is believed that the most important PI3Ks are those that belong to class IA, which contain a catalytic subunit p110α, and its associated regulatory subunit, p85α (1). In cells, p85α binds to p110α, stabilizing p110α and inactivating its kinase activity. Upon growth factor stimulation, the ShH2, (Rous sarcoma oncogene homology-2 domain) of p85 binds to phosphorylated tyrosine in receptor tyrosine kinase. This binding relieves the inhibition of p110α and mediates the recruitment of the catalytic subunit of PI3K to the plasma membrane (1, 13). PI3K activity is further augmented by the interaction between p110α and the activated form of Ras (14, 15). Although the molecular mechanisms underlying the regulation of class I PI3K are well characterized, the intracellular regulation of VPS34 remains elusive. hVPS34 has been previously identified as a downstream effector of Rab5, a Ras-like small GTP-binding protein (16). However, interaction between Rab5 and hVPS34 does not seem to modulate the lipid kinase activity of hVPS34 (16). pp60c-Src (Src) regulates many cellular functions by catalyzing the tyrosine phosphorylation of numerous substrates.

Authors’ Affiliation: Division of Monoclonal Antibodies, Office of Biotechnology Products, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland

Corresponding Author: Wen Jin Wu, Division of Monoclonal Antibodies, Office of Biotechnology Products, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, HFD-123, 29B Lincoln Drive, Room 3NN-15, Bethesda, MD 20892-4555. Phone: 301-827-0253; Fax: 301-827-0852; E-mail: wenwu@fda.hhs.gov.

doi: 10.1158/0008-5472.CAN-09-2682
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downstream of multiple signaling pathways including those regulated by insulin, insulin-like growth factor I, steroid hormone receptors, and epidermal growth factor (17, 18). Elevated protein levels and/or catalytic activity of Src have been detected in a number of human cancers, including breast, colon, lung, skin, ovarian, endometrial, and head and neck malignancies (19, 20). Several lines of evidence suggest that Src regulates mTOR complex 1/S6K1 downstream of PI3Ks (21–23). Although molecular connections between Src non–receptor tyrosine kinases and class I PI3Ks have been shown, signaling between Src and the class III PI3K, VPS34, has not been examined.

Here, we show that Src is an upstream regulator of hVPS34. We found that Src directly phosphorylates VPS34, and that this phosphorylation resulted in the activation of VPS34 to mediate cellular transformation. Furthermore, disruption of VPS34 function inhibits Src-Y527F–mediated cellular transformation in NIH3T3 cells.

Molecular cloning and generation of stable cell lines

pcDNA3–Myc-hVPS34 was a kind gift from Dr. Jonathan Backer. Myc-hVPS34–D747N, kinase–dead Myc-hVPS34–D747N, N748K (designated hVPS34–KD), Myc-hVPS34–Y231F, Myc–hVPS34–Y670F, and Myc–hVPS34–Y795F were generated by site-directed mutagenesis according to the protocols of the manufacturer (Stratagene). Src–Y527F–transformed NIH3T3 fibroblasts stably expressing HA–hVPS34–KD, HA–hVPS34–Y231F, or empty vector were generated by cotransfection of the indicated pl4–HA–hVPS34 constructs plus pcDNA3 empty vector as described previously (24–26). NIH3T3 Src–Y527F cells stably expressing either VPS34–targeting shRNA or control shRNA were generated under puromycin selection according to the protocols of the manufacturer (OriGene).

VPS34 small interfering RNA experiments

Cells were seeded in 12-well plates in triplicate at 1 × 10^5 cells per well and then transfected for 48 to 72 hours with pooled VPS34–targeting RNA duplexes (siGENOME SMART pool M-005250-00-0050; Thermo Scientific/Dharmacon) or control nontargeting small interfering RNA (siRNA; Thermo Scientific/Dharmacon).

Generation of rabbit anti-VPS34 polyclonal antibody

Rabbit anti-VPS34 antibody was initially obtained from Dr. Jonathan Backer. Subsequently, we generated rabbit anti-VPS34 polyclonal antibody against the hVPS34–specific immunogen (C)AVVEQIHKFAQYWYKR (hVPS34 residues 871–887; ref. 27).

Src in vitro kinase assays

COS-7 cells were transfected with Myc–hVPS34 for 48 hours followed by extraction and immunoprecipitation using anti-Myc antibody. Src in vitro kinase assays were conducted on immunoprecipitated Myc–hVPS34 according to the instructions of the manufacturer (Upstate).

VPS34 PI3K assays

COS-7 cells were transfected for 48 hours with different Myc–hVPS34 constructs plus either vector control or plasmid encoding Src–Y527F. Proteins were extracted and then Myc–hVPS34 proteins were immunoprecipitated using anti-Myc antibody. In vitro PI3K assays were performed at room temperature directly in the beads in the presence of 10 mmol/L of MnCl_2, 50 μmol/L of ATP, 15 μCi of γ-32P-ATP, 0.1 μg/μL of phosphatidylinositol, and 0.1 μg/μL of phosphatidylerine as previously described (28). PI3K assays using endogenous VPS34 immunoprecipitated from NIH3T3 or NIH3T3 cells stably expressing Src–Y527F were conducted as previously described (11). Spot densities of PI(3)P produced were determined by calculating the area under the curves using ImageJ software (29). PI3K activity was calculated by subtracting the area under the curve of the background signal (Ctrl) from the area under the curve of PI(3)P produced by VPS34. Data were normalized to PI(3)P produced by VPS34 and are presented as the mean ± SEM.

Immunofluorescence and microscopy

Cells were fixed and stained as previously described (30). Images were captured on an LSM 510 Meta confocal microscope attached to an Axiovert 200 inverted microscope using a 63×/1.40 numerical aperture objective (Carl Zeiss).

Statistical analyses

Data from soft agar assays and PI3K assays were analyzed using GraphPad Prism software and are presented as the mean ± SEM.

Results

VPS34 is tyrosine-phosphorylated in Src–Y527F–transformed NIH3T3 cells

To identify upstream regulators of VPS34, we screened for the status of VPS34 tyrosine phosphorylation in protein extracts from NIH3T3 cells, NIH3T3 cells stably expressing Cdc42ΔL8/F28L, and NIH3T3 cells transformed by stable expression of Cdc42–F28L, Ras–G12V, or Src–Y527F (24–26). We found that endogenous VPS34 was only tyrosine-phosphorylated in Src–Y527F–transformed NIH3T3 cells (Fig. 1A). Ectopically expressed Myc–hVPS34 was tyrosine-phosphorylated in Src–Y527F–transformed cells, but not in NIH3T3 parental cells (Fig. 1B). Tyrosine phosphorylation of endogenous VPS34 detected in Src–Y527F–transformed cells was significantly reduced by the Src-specific inhibitor, SU6656 (Fig. 1C). Additionally, VPS34 protein levels were higher in Src–Y527F–transformed NIH3T3 cells as compared with NIH3T3 cells (Fig. 1D). Taken together, these data suggest that both tyrosine phosphorylation and protein expression of VPS34 are regulated by Src.

VPS34 is required for Src-induced cellular transformation

Class I PI3Ks are known to contribute to tumorigenesis (1, 31). However, VPS34 has not been studied in this context. To determine if VPS34 plays a role in the regulation...
of Src-induced cellular transformation, we tested whether silencing endogenous VPS34 expression affected Src-Y527F-induced cellular transformation. Transfection with VPS34-targeting siRNA resulted in a 50% knockdown of VPS34 protein levels in both Src-Y527F and Ras-G12V transformed cells (Fig. 2A and B, Western blots). We observed a >10-fold decrease in colony formation in soft agar growth assays in Src-Y527F–transformed cells transfected with VPS34-targeting siRNA as compared with nontargeting siRNA (Ctrl; Fig. 2A). In contrast, colony formation in Ras-G12V–transformed cells was not inhibited (Fig. 2B). Micrographs in Fig. 2A and B represent growth in soft agar observed at 2 weeks after siRNA transfection. To confirm that decreasing VPS34 protein levels interfere with Src-mediated cellular transformation, we stably expressed either control shRNA or VPS34-targeting shRNA in Src-Y527F–transformed cells. Two clones (110-7 and 110-15) with an ~50% decrease in VPS34 protein levels were evaluated for growth in soft agar and found to inhibit colony formation by >50% as compared with the control cell line (Fig. 2C). Additionally, reduced expression of VPS34 resulted in flattening of Src-Y527F–transformed cells (Fig. 2D, top). Figure 2D (bottom) shows representative micrographs of the growth in soft agar observed at 2 weeks. These data indicate that VPS34 is required for Src-induced cellular transformation.

**Src phosphorylates hVPS34**

Based on the above data, we conducted experiments to determine if VPS34 is a direct Src substrate. Myc-hVPS34 was only tyrosine-phosphorylated when coexpressed with Src-Y527F (Fig. 3A). We also observed that protein levels of Myc-hVPS34 were decreased in cells coexpressing the Src kinase-dead mutant, Src-R295M, Y527F (Src-DN), as compared with cells either expressing Myc-hVPS34 alone or coexpressing Myc-hVPS34 and Src-Y527F. Next, we performed in vitro kinase assays using immunoprecipitated Myc-hVPS34 and recombinant Src and found that hVPS34 was phosphorylated by recombinant Src (Fig. 3B). Immunoprecipitation of Myc-hVPS34 was confirmed by Western blot analysis of the immunoprecipitated Myc-hVPS34 used in the in vitro kinase reactions. These data indicate that hVPS34 is a Src substrate. We additionally found that in COS-7 cells cotransfected with Myc-hVPS34 and Src-Y527F, Src-Y527F coimmunoprecipitated with ectopically expressed Myc-hVPS34, and that in NIH3T3-Src-Y527F cells, Src coimmunoprecipitated with endogenous VPS34 (Fig. 3C).

**Phosphorylation of VPS34 by Src results in the activation of VPS34 lipid kinase activity**

We next determined if Src regulates the PI3K activity of hVPS34. Myc-hVPS34 was coexpressed with either empty vector or Src-Y527F. In vitro PI3K assays were conducted using immunoprecipitated Myc-hVPS34 as previously described (28). As shown in Fig. 4A, lipid kinase activity of hVPS34 as measured by the production of PI(3)P was significantly enhanced in cells coexpressing Myc-hVPS34 and Src-Y527F as compared with cells expressing either vector control or Myc-hVPS34 alone. Based on Western blot analysis, levels of immunoprecipitated Myc-hVPS34 used for the in vitro PI3K assays were comparable (Fig. 4A, middle). Endogenous VPS34 PI3K activity was also higher in Src-Y527F cells as compared with NIH3T3 cells (Fig. 4B). As shown in Fig. 4C, endogenous VPS34 bound to VPS15 in both NIH3T3 and Src-Y527F–transformed cells, which indicates that the increased lipid kinase activity observed in Src-Y527F–transformed cells was independent of VPS34 binding to VPS15 (Fig. 4C).

**Kinase-dead mutations in hVPS34 reduce Src-catalyzed hVPS34 tyrosine phosphorylation**

Yeast Vps34p is a multiple-specificity kinase that exhibits both protein kinase autophosphorylation and PI3K activities (3). Mutations have been identified in yeast Vps34p that
abolish its lipid kinase and protein kinase activities (3). We generated two kinase domain mutants in hVPS34, hVPS34-D747N and hVPS34-D747N, N748K (designated hVPS34-KD), analogous to the kinase-disrupting mutations identified in yeast Vps34p. We then determined whether the lipid kinase activity of hVPS34 was disrupted by the hVPS34-KD mutation. As depicted in Fig. 5A, the lipid kinase activity of hVPS34-KD as measured by the production of PI(3)P was significantly diminished. Interestingly, we also found that Src-catalyzed tyrosine phosphorylation of hVPS34 was significantly reduced in the hVPS34 kinase domain mutants as compared with wild-type hVPS34 (Fig. 5B, compare lane 3 to lanes 6 and 7). Furthermore, SU6656 blocked Src-catalyzed tyrosine phosphorylation of Myc-hVPS34 in COS-7 cells (lane 4), which is consistent with the observation shown in Fig. 1C. Notably, hVPS34 expression was always reduced when expressed together with Src-DN (lane 2).

**hVPS34-Y231F point mutant decreased Src-catalyzed hVPS34 tyrosine phosphorylation**

We next focused on identifying sites of Src-catalyzed hVPS34 tyrosine phosphorylation. A point mutation at tyrosine 670 had no effect on hVPS34 tyrosine phosphorylation by Src (Fig. 5B, lane 5). We then created two additional single point mutations at tyrosine residues 231 and 795, which were predicted by the "KinasePhos" web tool to be Src phosphorylation sites (32). Of these two sites, only the point mutation introduced at tyrosine 231 (hVPS34-Y231F) reduced Src-catalyzed tyrosine phosphorylation of hVPS34 (Fig. 5B, bottom four panels). Because tyrosine phosphorylation levels were only reduced by ∼50%, these data suggest that there might be multiple Src phosphorylation sites in hVPS34. We next examined the effect of the Y231F mutation on hVPS34 PI3K activity. As shown in Fig. 5C, the intrinsic PI3K activity of Myc-hVPS34-Y231F as measured by the production of PI3K activity.
In an in vitro PI3K assay was slightly reduced as compared with that of Myc-hVPS34. However, whereas wild-type hVPS34 lipid kinase activity was increased when Src-Y527F was coexpressed, no increase in PI(3)P production was observed when hVPS34-Y231F was coexpressed with Src-Y527F. These data suggest that phosphorylation at Y231 is necessary for Src to positively regulate hVPS34 lipid kinase activity. Averaged PI(3)P production, normalized to that of Myc-hVPS34 as described in Materials and Methods, is presented in Fig. 5D.

**Src-mediated cellular transformation is inhibited by stable overexpression of hVPS34 mutants**

Given that hVPS34 is a Src substrate and lipid kinase activity of VPS34 is upregulated by Src-catalyzed tyrosine phosphorylation, we asked whether hVPS34-KD or hVPS34-Y231F would affect Src-induced cellular transformation. We stably expressed HA-hVPS34-KD and HA-hVPS34-Y231F in Src-Y527F–transformed NIH3T3 cells. Protein expression of HA-hVPS34-KD (clones KD-14 and KD-29) and HA-hVPS34-Y231F (clones Y231F-33 and Y231F-34) in Src-Y527F-transformed cells are shown in Fig. 6A. Although Src-Y527F cells stably expressing empty vector (V-15) grew in low serum medium, growth in low serum was significantly inhibited in hVPS34-KD clones, KD-14 and KD-29 (Fig. 6A, graph). Growth under low serum conditions was not inhibited in the Y231F-33 and Y231F-34 clones (data not shown). In soft agar assays, colony formation in KD-14 and KD-29 cells was reduced 4-fold as compared with that in V-15 cells (Fig. 6B, left graph). When only colonies ≥100 μm were scored, clone KD-14 formed ∼6-fold fewer colonies as compared with V-15 control cells, and no colonies >100 μm in size were detected in KD-29 cells (Fig. 6B, right graph).

A comparison of growth in soft agar between V-15 cells and clones Y231F-33 and Y231F-34 cells indicated dose-dependent inhibition of colony formation, such that colony formation (percentage of colonies in size ≥50 μm) was decreased ∼40% in Y231F-33 cells and 60% in Y231F-34 cells (Fig. 4B, left graph). When only colonies ≥100 μm were scored as positive, Y231F-33 formed 3-fold fewer colonies and Y231F-34 formed 6-fold fewer colonies as compared with V-15 control cells (Fig. 6B, right graph). This suggests that Src phosphorylation of hVPS34 at tyrosine 231 is required for Src-Y527F–mediated transformation of NIH3T3 cells. In addition, we observed that overexpression of hVPS34-KD caused an increased number of binucleate and multinucleate cells, suggesting that the kinase activity of hVPS34 is required for cytokinesis. This observation is consistent with a recent report (Fig. 6C; ref. 33).

**Figure 3.** hVPS34 is a Src substrate. A, COS-7 cells were transfected with Myc-hVPS34 plus empty vector, Src-R295M,Y527F (Src-DN), or constitutively active Src (Src-Y527F). Following anti-Myc immunoprecipitation, immunoprecipitates were probed with 4G10. The blot was then stripped and reprobed with anti-Myc antibody. Ectopically expressed Myc-hVPS34 and Src proteins were detected in WCL by Western blot. B, Myc-hVPS34 was immunoprecipitated from COS-7 cells in vitro kinase assays were conducted on immunoprecipitated Myc-hVPS34 directly on the beads. The kinase assay was stopped using Laemli buffer. Samples were resolved by SDS-PAGE. γ-32P–labeled hVPS34 was detected by autoradiography. Blots were then probed with anti-Myc antibody to detect immunoprecipitated Myc-hVPS34. C, anti-Src, anti-Myc, and control IgG immunoprecipitations were done using protein extracts from COS-7 cells cotransfected with Myc-hVPS34 and Src-Y527F. Western blots were probed for VPS34 and Src. Data are representative of two independent experiments.
The inhibition of Src-induced cellular transformation by expression of the hVPS34 mutants and the upregulation of VPS34 protein and tyrosine phosphorylation in Src-Y527F-transformed cells led us to explore whether VPS34 protein levels were increased in human breast cancer cell lines as compared with the nontumorigenic breast epithelial cell line (MCF-10A). hVPS34 protein levels were examined from protein extracts of high tumorigenic cell lines, MDA-MB-231, MDA-MB-468, SKBR-3, and relatively low tumorigenic cell lines, MCF-7 and T47D. hVPS34 protein levels were significantly elevated in SKBR-3, MDA-MB-231, and MDA-MB-468 breast cancer cell lines as compared with MCF-7 cells and MCF-10A cells (Fig. 6D). Additionally, VPS34 tyrosine phosphorylation was detected in high tumorigenic MDA-MB-231 cells and MDA-MB-468 cells, but not in MCF-7 cells (Fig. 6D, bottom three panels).

Discussion

VPS34 was first described as a component of the vacuolar sorting system in Saccharomyces cerevisiae. Although VPS34 has been studied extensively in the context of endocytic sorting, and recently has been implicated in regulating nutrient signaling as well as the mTOR/S6K1 pathway (2, 10, 11), it still remains elusive how its lipid kinase activity is regulated in cells. Although Vps15p has been described as a Vps34p regulatory protein, the sequence of Vps15p suggests that it functions as a protein kinase (2) regulatory protein. In yeast, Vps34p activity requires a functional copy of Vps15p, which binds Vps34p to form a membrane-associated complex (34). In addition, Vps34p has been found phosphorylated in yeast, but this phosphorylation most likely reflects autoprophosphorylation rather than phosphorylation by Vps15p (3). Furthermore, the lipid kinase activity of yeast Vps34p is minimally affected by partial dephosphorylation with potato acid phosphatase (3). Toward this end, one of the important questions that has been raised in this research field is whether Vps34p is regulated by phosphorylation (2). It is believed that the lipid kinase in Vps34p might be constitutively activated (1).

Here, we describe a novel molecular mechanism by which Src tyrosine phosphorylates VPS34 in mammalian cells and this phosphorylation results in the enhancement of VPS34 lipid kinase activity. First, this finding provides important evidence that the lipid kinase activity of VPS34 can be upregulated by tyrosine phosphorylation in mammalian cells. Second, our data highlight a previously unappreciated relationship between VPS34 and Src non–receptor tyrosine kinase. Our results show that binding of VPS15 to VPS34 in NIH3T3 cells neither affects the Src-catalyzed tyrosine phosphorylation of VPS34 nor the lipid kinase activity of VPS34 because VPS34 binds to VPS15 equally well in both NIH3T3 cells and Src-Y527F-transformed NIH3T3 cells (Figs. 1 and 4). Further characterization is needed to determine whether VPS15 is required for Src-mediated tyrosine phosphorylation and activation of VPS34 in mammalian cells. The data presented here suggest that Src-catalyzed tyrosine

Figure 4. Src upregulates VPS34 PI3K activity. A, COS-7 cells were transiently transfected with empty vector (Ctrl), or Myc-hVPS34 with or without Src-Y527F. In vitro PI3K assays using phosphatidylinositol as a substrate were conducted directly on beads containing anti-Myc immunoprecipitated hVPS34. Phospholipids were resolved by TLC and radiolabeled PI(3)P was detected by autoradiography. Data are representative of three independent experiments (see Fig. 5D for quantitative results). Protein expression was confirmed by Western blot analysis. B, in vitro PI3K assays of endogenous VPS34 immunoprecipitated from NIH3T3 cells and Src-Y527F NIH3T3 cells were conducted as described in Materials and Methods. For the control lane, WCL of Src-Y527F-transformed cells was incubated with rabbit IgG control antibody. Histogram represents the mean of two independent experiments + SEM. C, endogenous VPS34 was immunoprecipitated from the indicated cell lines. VPS34 and VPS15 were detected by Western blot analysis using their respective antibodies.
phosphorylation of VPS34 directly activates VPS34 lipid kinase activity, although it is possible that Src phosphorylation of VPS34 affects VPS34 interactions with other VPS34-activating proteins.

The data presented here indicate that VPS34 is a direct substrate of Src kinase. However, it is likely that VPS34 has multiple tyrosine phosphorylation sites as Src-catalyzed phosphorylation of hVPS34 is only partially reduced by Src-Y527F–catalyzed tyrosine phosphorylation of hVPS34.

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the mutation at Y231F. This partial reduction in hVPS34 tyrosine phosphorylation inhibits Src-mediated upregulation of hVPS34 lipid kinase activity, which likely contributes to the reduction in Src-Y527F–mediated growth in soft agar observed in Src-Y527F cells stably expressing hVPS34-Y231F. This has led us to propose a mechanism by which maximal tyrosine phosphorylation of VPS34 by Src is required for Src to positively regulate VPS34 lipid kinase activity. We have also found that the protein levels of VPS34 are increased in Src-Y527F–transformed cells, which could be either due to increased protein stability of VPS34 when it is tyrosine-phosphorylated or upregulation of VPS4.

Figure 6. Src-mediated cellular transformation is disrupted by stable overexpression of hVPS34-KD and hVPS34-Y231F mutants. A, pJ4-HA-hVPS34-KD, pJ4-HA-hVPS34-Y231F, and pcDNA3-neo empty vector were stably transfected into Src-Y527F–transformed NIH3T3 cells. Stable cell line selection was achieved using G418. Expression levels of the different HA-hVPS34 proteins were determined by Western blot analysis using anti-HA antibody. V-15, KD-14, and KD-29 cells were seeded at 5 x 10⁴ cells per well in duplicate in DMEM supplemented with 1% calf serum. Cells were counted on days 2, 4, and 6. Data are the average from two independent experiments (bars, SD). B, the indicated stable cell lines were seeded in soft agar. Colony formation was quantified on day 7. Colonies ≥50 or ≥100 μm were scored for growth in soft agar. Data are the mean of three to five independent experiments. Data were evaluated by ANOVA followed by the Dunnett’s test (*, P < 0.05; bars, SEM). C, the indicated cell lines were seeded on slides overnight and then fixed and permeabilized. Cells were stained using anti-PI(3)P antibody. Open arrows, punctate staining of PI(3)P in V-15 vector control cells. Closed arrow, multinucleated cells in KD-29 cells (magnification, ×1,200). Representative phase contrast images of V-15 control cells and KD-29 cells (magnification, ×200). D, the indicated breast cancer cells were seeded overnight, harvested, and then WCL was subjected to immunoprecipitation using anti-VPS34 antibody. Immunoprecipitated VPS34 was detected by Western blot (top). Bottom, endogenous VPS34 was immunoprecipitated from the indicated cell lines. Western blots were probed with antiphosphotyrosine antibody and then reprobed for VPS34. Rabbit IgG immunoprecipitation using MDA-MB-231 WCL was run as a control.
expression. Nevertheless, this increase in VPS34 protein levels may confer enhanced proliferative signals to downstream signaling pathways, such as mTOR/S6K1, to mediate Src-induced cellular transformation.

According to the recently published crystal structure of VPS34, amino acid N748 in VPS34 is part of the catalytic loop (35). Consistent with this finding, hVPS34-D747N, N748K mutant abolished lipid kinase activity. Interestingly, our data show that point mutations in the kinase domain of hVPS34 not only abolish hVPS34 lipid kinase activity, but also reduce Src-catalyzed VPS34 tyrosine phosphorylation. This reduction in Src-catalyzed tyrosine phosphorylation might be due to conformational changes that hinder Src interaction with substrate residues in hVPS34.

The present finding highlights the role of VPS34 in the regulation of cell proliferation and Src-mediated cellular transformation. Our data show that the lipid kinase activity of VPS34 is essential for Src-Y527F–induced cellular transformation. VPS34 is upstream of mTOR complex 1/S6K1 signaling, which is a critical growth control node, receiving stimulatory signals from insulin and nutrient inputs (31). Upregulation and/or constitutively active mutations in class I PI3K, loss of PTEN, and upregulation of S6K1 have all been identified in specific cancers (12, 36). Our data suggest that the levels of VPS34 protein expression and tyrosine phosphorylation are correlated with the tumorigenic activity of human breast cancer cells, suggesting that VPS34 may be involved in human breast cancer progression. Data presented here indicate that Src to VPS34 signaling warrants further investigation as a pathway contributing to the development and progression of human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Dr. Jonathan M. Backer of Albert Einstein College of Medicine (Bronx, NY) for providing rabbit polyclonal anti-VPS34 antibodies and the Myc-VPS34 construct. We thank Drs. Michele K. Dougherty and Caryl J. Giuliano for critical review of this manuscript. The information presented in this article reflects the views of the authors and does not necessarily represent the policy of the U.S. Food and Drug Administration.

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Received 07/20/2009; revised 04/26/2010; accepted 04/29/2010; published OnlineFirst 06/15/2010.

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pp60c-Src Phosphorylates and Activates Vacuolar Protein Sorting 34 to Mediate Cellular Transformation


Cancer Res 2010;70:5974-5983. Published OnlineFirst June 15, 2010.

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