Phosphatidylinositol 4-Phosphate in the Golgi Apparatus Regulates Cell–Cell Adhesion and Invasive Cell Migration in Human Breast Cancer

Emi Tokuda¹, Toshiki Itoh², Junya Hasegawa², Takeshi Ijuin¹, Yukiko Takeuchi¹, Yasuhiro Irino³, Miki Fukumoto¹, and Tadaomi Takenawa¹

Abstract
Downregulation of cell–cell adhesion and upregulation of cell migration play critical roles in the conversion of benign tumors to aggressive invasive cancers. In this study, we show that changes in cell–cell adhesion and cancer cell migration/invasion capacity depend on the level of phosphatidylinositol 4-phosphate [PI(4)P] in the Golgi apparatus in breast cancer cells. Attenuating SAC1, a PI(4)P phosphatase localized in the Golgi apparatus, resulted in decreased cell–cell adhesion and increased cell migration in weakly invasive cells. In contrast, silencing phosphatidylinositol 4-kinase IIIβ, which generates PI(4)P in the Golgi apparatus, increased cell–cell adhesion and decreased invasion in highly invasive cells. Furthermore, a PI(4)P effector, Golgi phosphoprotein 3, was found to be involved in the generation of these phenotypes in a manner that depends on its PI(4)P-binding ability. Our results provide a new model for breast cancer cell progression in which progression is controlled by PI(4)P levels in the Golgi apparatus. Cancer Res; 74(11); 3054–66. ©2014 AACR.

Introduction
Phosphoinositides are lipid constituents of plasma and organelle membranes and comprise seven different phosphor-ylated versions. The levels of various phosphorylated phosphoinositides within the cell are regulated by multiple phosphoinositide kinases and phosphoinositide phosphatases (1, 2). Phosphatidylinositol 4-phosphate [PI(4)P] is a relatively abundant phosphoinositide required for the maintenance and function of the Golgi apparatus, including intracellular trafficking (3). The PI(4)P level at the Golgi apparatus is controlled by phosphatidylinositol 4-kinases (PHK) and SAC1 phosphoinositide phosphatase. The PI(4)P isoforms phosphatidylinositol 4-kinase IIc (PHIKIIC) and PHIKIIβ are known to localize to the Golgi apparatus and to be involved in membrane transport from the trans-Golgi network to the plasma membrane (4, 5). In contrast, SAC1, which dephosphorylates PI(4)P at the Golgi apparatus, is involved in the same membrane transport pathway and in protein glycosylation (6).

Several PI(4)P-binding proteins, including FAPP, OSBP, CERT, and GGA, have been identified (7). One PI(4)P effector, Golgi phosphoprotein 3 (GOLPH3), maintains a tensile force on the Golgi apparatus and plays a role in the Golgi secretory pathway (8, 9). Recently, GOLPH3 was shown to be involved in cancer progression. Human chromosome 5p13, on which the GOLPH3 gene is located, is amplified in several cancers (10), and expression of GOLPH3 is increased in several cancer cell lines and in some patients with cancer (11–14). GOLPH3 enhances cell proliferation and tumorigenicity (10, 15). However, its role in cell migration and invasion remains unknown.

To metastasize from their location within a primary tumor to the surrounding tissue, tumor cells undergo several processes, including loss of cell–cell adhesion and gains in motile and invasive potential (16). This temporary and reversible phenotype is referred to as epithelial–mesenchymal transition (EMT). Following invasion or distal metastasis, metastatic tumor cells revert to an epithelial phenotype in the so-called mesenchymal–epithelial transition (MET). EMT/MET conversions are recognized as critical events in cancer progression (17).

To identify the physiologic role of PI(4)P in the Golgi apparatus during tumor progression, we investigated the effects of PI(4)P metabolism on cell–cell adhesion and cell migration in breast cancer cells. PI(4)P in the Golgi apparatus was found to regulate cell–cell adhesion, cell migration, invasion, and metastasis, which are essential for breast cancer progression through regulation of GOLPH3 localization to the Golgi apparatus. On the basis of these results, we propose a novel mechanism for the development of cancer progression.
Materials and Methods

Reagents and antibodies

Mouse anti–E-cadherin, β-catenin, P4KIIIβ, and GM130 monoclonal antibodies were purchased from BD Biosciences. Rat anti–E-cadherin monoclonal, mouse anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal, rabbit anti-SAC1, rabbit anti–GOLPH3, and anti-cadherin 11 antibodies were purchased from Takara Bio, Sigma-Aldrich, Protein-tech, Epitomics, and Cell Signaling Technology, respectively. All fluorescent reagents and antibodies were purchased from Life Technologies. Purified phosphatidylethanolamine and phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. Purified phosphoinositides were obtained from CellSignals Inc. and human breast cancer tissue lysates from OriGene Technologies.

Plasmids

Human Fapp1 2 × PH, Grp1 PH, PLCδ1 PH, and Hrs1 FYVE cDNA fragments were subcloned into the pGEX-6P1 vector (GE Healthcare) as described previously (18). GOLPH3 was generated by PCR amplification with MDA-MB-231 cDNA as a template and subcloned into the pRetroQ-AcGFP1-C1 vector (Clontech Laboratories, Inc.). The GOLPH3 R90L mutant was generated from GOLPH3-wt DNA using site-directed mutagenesis.

Cell culture

The human breast cancer cell lines MCF7, MDA-MB-23, and Hs578t and GP2-293 were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS. T-47D cells were maintained in RPMI medium supplemented with 10 μg/mL insulin and 10% FBS. The cell lines were obtained from the American Type Culture Collection. The authenticity of all cell lines was confirmed by short tandem repeat profiling by National Institute of Biomedical Innovation in Japan. For treatment of TGFβ (WAKO) and TNFα (WAKO), MCF7 cells were treated with 2 ng/ml TGFβ and 5 ng/ml TNFα for 72h.

RNA interference

Stealth siRNAs targeting phosphoinositide phosphatases, SAC1 (HSS117880 and HSS117881), P4KIIIβ (HSS108028 and HSS108029), and GOLPH3 (HSS127330 and HSS127331) were purchased from Life Technologies. The stealth siRNAs were transfected into cells with Lipofectamine RNAiMAX (Life Technologies); experiments were performed 72 hours after transfection.

Production of retrovirus-infected cell lines

Retroviruses with the VSV-G envelope were packaged and produced according to manufacturer’s instruction (Clontech Laboratories, Inc.). Cells were infected with retroviruses in the presence of 8 μg/mL polybrene. The infected cells were selected by adding 1 μg/mL puromycin to the medium for 2 weeks.

Immunofluorescence analysis

Cells grown on coverslips were fixed with 3.7% formaldehyde in PBS and permeabilized using 0.2% Triton X-100 or 20 μmol/L digitonin in PBS and blocked with 1.5% BSA in PBS before
Figure 2. SAC1 knockdown decreases cell-cell adhesion and promotes cell migration. A, the indicated siRNA-transfected MCF7 cells were lysed and immunoblotted with the indicated antibodies. B, the indicated siRNA-transfected MCF7 cells were fixed and stained with anti-E-cadherin antibody and phalloidin. Scale bar, 20 μm. C, the indicated siRNA-transfected MCF7 cells were fractionated into Triton X-100-soluble and Triton X-100-insoluble fractions. Each fraction was immunoblotted with the indicated antibodies. D, the indicated siRNA-transfected MCF7 cells were analyzed using a migration assay. The data shown are the mean (SEM) values of three independent experiments. *, P < 0.001; **, P < 0.005. Scale bar, 100 μm. E, MCF7 cells were transfected with the indicated siRNAs. RhoA activity was measured using a G-LISA RhoA activation assay. The data shown are the mean (SEM) values of four independent experiments. **, P < 0.01. F, MCF7 cells were transfected with indicated siRNAs. The SNAI2 mRNA level was quantified by real-time PCR. The data shown are the mean (SEM) values of three independent experiments. **, P < 0.003. G, Golgi PI(4)P levels were quantified in the indicated siRNA-transfected MCF7 cells. The data shown are the mean (SEM) values from more than 100 cells measured. **, P < 0.001. H, the indicated siRNA-transfected MCF7 cells were fixed and stained with the anti-GM130 antibody and Alexa Fluor 647–labeled Fapp1 2 × PH domain. Scale bar, 20 μm.
incubation with primary antibodies diluted in blocking buffer. Immunofluorescence analysis was performed as previously described (19).

Quantification of Golgi PI(4)P levels
Recombinant GST-conjugated proteins were expressed in the Rosetta-gami B(DE3)pLyS bacterial strain (Merck) and purified on glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. GST was removed by on-column cleavage with PreScission protease (GE Healthcare). Recombinant Fapp1 2× PH domain protein was labeled using the Alexa Fluor 647 Protein Labeling Kit (Life Technologies) and used as a fluorescent probe for detecting Golgi PI(4)P. Cells were fixed with 3.7% formaldehyde in PBS, permeabilized using 20 μmol/L digitonin in PBS, and stained as described under “immunofluorescence analysis.” PI(4)P levels were quantified using FluoView software. For quantifying the amount of PI(4)P at the Golgi apparatus, the fluorescence intensity of AF647-labeled Fapp1 2× PH was normalized to that of GM130, a Golgi apparatus marker.

Matrigel invasion assay
The indicated siRNA-transfected or the indicated retrovirus-infected MDA-MB-231 cells were plated in a BioCoat Matrigel invasion chamber (BD Biosciences) and cultured for 18 hours. The cells were fixed with 3.7% formaldehyde in PBS, and the invading cells were stained with phalloidin.

Migration assay
The indicated siRNA-transfected MCF7 or T-47D cells, or the indicated retrovirus-infected MCF7 cells were seeded. Forty-eight hours after transfection or seeding, the cells were serum starved for 16 hours. The migration assay was performed for 24 hours.

Figure 3. EMT-like phenotypes are affected by PI(4)P levels in the Golgi apparatus. A, MCF7 cells, not treated or treated with 2 ng/mL TGFβ + 5 ng/mL TNFα, were fixed and stained with anti-GM130 antibody together with Fapp1 2× PH domain. Scale bar, 20 μm. B, quantification of Golgi PI(4)P levels in MCF7 cells before and after EMT induction. The data shown are the mean (SEM) values from more than 100 cells. * P < 0.001. C, each breast cancer cell line was fixed and stained with anti-GM130 antibody and the Alexa Fluor 647–labeled Fapp1 2× PH domain. Scale bar, 20 μm. D, Golgi PI(4)P levels were quantified in breast cancer cell lines. The data shown are the mean (SEM) values from more than 100 cells measured. * P < 0.001. E, human breast cancer tissue lysates were immunoblotted with the antibodies against PI4KIIIβ and SAC1. Lysates isolated from four nonmetastatic and four metastatic breast cancer subjects were tested.
Figure 4. PI4KIIIβ knockdown induces cell–cell adhesion and suppression of invasion. A, the indicated siRNA-transfected MDA-MB-231 cells were fixed and stained with anti-GM130 antibody and Alexa Fluor 647–labeled Fapp1 2 × PH domain. Scale bar, 20 μm. B, Golgi PI(4)P levels were quantified in control- or PI4KIIIβ-silenced MDA-MB-231 cells. The data represent the mean (SEM) values from more than 100 cells. * * 0.001. (Continued on the following page.)
Subcellular fractionation

Cells were resuspended in Triton X-100 buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2% glycerol, 0.1% Triton X-100, 1 mmol/L CaCl₂, and Complete Mini (Roche Diagnostics)]. The cell lysate was centrifuged at 20,630 × g for 15 minutes at 4°C. The supernatant was collected as a Triton X-100 soluble fraction. The pellet was sonicated in SDS buffer [20 mmol/L Tris-HCl (pH 7.5), 1% SDS, and 10% glycerol] and collected as a Triton X-100 insoluble fraction.

Real-time PCR

Total RNA was isolated from cells using TRIzol (Life Technologies) and used as a template for cDNA synthesis with the PrimeScript II First-Strand cDNA Synthesis Kit (Takara). Real-time PCR was performed using a TaqMan probe in an ABI 7500 real-time PCR system (Life Technologies). The data were normalized to those obtained for GAPDH.

RhoA activation assay

Cells were transfected with the indicated siRNAs. Seventy-two hours after transfection, a RhoA activation assay was performed using a G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton, Inc.). The data were normalized to total RhoA.

Tumor tail vein injection

Experiments were performed with 7-week-old female BALB/c-nu/nu mice (Charles River Laboratories). All experiments were approved by the Institutional Animal Care and Use Committee in Kobe University and were carried out in accordance with the institute’s guidelines. Cells suspended in Hanks’ Balanced Salt Solution (Gibco) were injected intravenously (1 × 10⁶ cells/mouse). Three weeks after injection, the mice were euthanized and their lungs were fixed with Bouin’s Fluid (Wako Pure Chemical Industries). The number of lung surface metastatic foci was counted.

Results

Effect of knockdown of various phosphatases on cell–cell adhesion

Noninvasive MCF7 human breast cancer cells normally exhibit tight cell–cell adhesion. To obtain insights into the role of phosphoinositide metabolism in the regulation of cell–cell adhesion, we investigated the effect of siRNA-mediated depletion of all phosphoinositide phosphatase isoforms in MCF7 cells (Fig. 1). Among siRNAs targeting 22 phosphoinositide phosphatases, transfection of those targeting PTEN, INPP4B, SAC1, INPP5D (SHIP1), and PIB5PA (PIPP) resulted in decreases in cell adhesion (Fig. 1, asterisk). PTEN, SAC1, INPP5D, and PIB5PA have been previously reported to exhibit tumor-suppressive activity through dephosphorylation of PI(3,4,5)P₃ or PI(3,4)P₂ and subsequent inhibition of PI3K signaling (20–22).

SAC1 knockdown increases PI(4)P levels in the Golgi apparatus and affects cell–cell adhesion and cell migration

SAC1, which dephosphorylates PI(4)P to PI in the Golgi apparatus, has not been reported to be involved in cancer progression. Transfection of MCF7 cells with SAC1-targeting siRNA did not alter E-cadherin or β-catenin protein levels in these cells (Fig. 2A), although it did result in reduced cell–cell adhesion (Fig. 2B). Similar results were observed in T-47D human ductal breast epithelial tumor cells, which normally exhibit cell–cell adhesion (Supplementary Fig. S1A and S1B). We therefore used subcellular fractionation to monitor the association of the actin cytoskeleton with E-cadherin and β-catenin in these cells. The amount of E-cadherin and β-catenin in the Triton X-100 insoluble fraction decreased in SAC1-depleted cells (Fig. 2C). Consistent with the previous finding that cell migration increases as a consequence of the disappearance of cell–cell adhesion (23), SAC1 depletion promoted MCF7 cell (Fig. 2D) and T-47D cell (Supplementary Fig. S1C) migration and caused reduction in cell–cell adhesion in both cell types. SAC1 depletion seemed to induce stress fiber formation at the cell periphery in MCF7 cells (Supplementary Fig. S3A), with an increase in RhoA activity in these cells (Fig. 2E). Furthermore, SAC1 depletion induced expression of SNAI2, an EMT marker in MCF7 cells (Fig. 2F). These results indicate that SAC1 partly regulates EMT, which is characterized by changes in the localization of E-cadherin and β-catenin at cell–cell adhesion sites, actin cytoskeletal rearrangement, and expression of EMT markers.

Because Sac1 dephosphorylates PI(4)P in the Golgi apparatus, we quantified the levels of cellular phosphoinositides using a liposome overlay assay. No change in the total amount of phosphoinositides was detected in Sac1-depleted cells (Supplementary Fig. S2A and S2B). Therefore, we measured relative PI(4)P levels in the Golgi apparatus using Alexa Fluor 647 (AF647)-labeled Fapp1 PH domains (Fapp1 2 × PH) that specifically recognize PI(4)P with high affinity (18). PI(4)P localized at the Golgi apparatus of HeLa cells can be efficiently detected by this fluorescent probe after permeabilization of the cells with digitonin (Supplementary Fig. S2C; ref. 24). The specificity of the fluorescence signals was confirmed by preincubation of the probe with PC/PE/PI(4)P liposomes, which completely blocked PI(4)P detection in the Golgi apparatus (Supplementary Fig. S2C). Using this method, we found that...
Figure 5. GOLPH3 is required for regulation of cell–cell adhesion and for migration/invasion ability. A, MDA-MB-231 cells were transfected with control or GOLPH3 siRNA and then fixed and stained with anti-GOLPH3 and anti-GM130 antibodies, and phalloidin. Scale bar, 20 μm. B, the indicated siRNA-transfected MDA-MB-231 cells were lysed and immunoblotted with GOLPH3 antibodies. C, the indicated siRNA-transfected MDA-MB-231 cells were fixed and stained with anti-β-catenin antibody and phalloidin. Scale bar, 20 μm. D, the indicated siRNA-transfected MDA-MB-231 cells were seeded onto Matrigel invasion chambers. Eighteen hours after seeding, the cells were fixed and stained with phalloidin (top). The number of invasive cells that passed through each Matrigel chamber was counted (bottom). The data shown are the mean (SEM) values of three independent experiments. *, *P < 0.001. Scale bar, 100 μm. E, the indicated siRNA-transfected MCF7 cells were analyzed in a migration assay. The data shown are the mean (SEM) values of four independent experiments. *, *P < 0.01; **, *P < 0.005. Scale bar, 100 μm.
Sac1 knockdown increased PI(4)P levels in the Golgi apparatus by approximately 2-fold, compared with that in control MCF7 cells (Fig. 2G and H). Further knockdown of PI4KIIIβ, which generates PI(4)P in the Golgi apparatus, in SAC1-depleted MCF7 cells restored the formation of cell–cell adhesion (Supplementary Fig. S3B and S3C), indicating that PI(4)P promotes cell migration by disrupting cell–cell adhesion.

PI(4)P levels in the Golgi apparatus during breast cancer progression

TGFβ and TNFα are known to induce EMT in several cancer cell lines (25, 26). Treatment with TGFβ and TNFα efficiently altered the normal epithelial morphology of MCF7 cells, producing a migratory phenotype. TGFβ- and TNFα-treated MCF7 cells also exhibited an increase of approximately 2-fold in PI(4)P levels in the Golgi apparatus (Fig. 3A and B). We then compared PI(4)P levels at the Golgi apparatus in highly invasive (MDA-MB-231 and Hs578t) and weakly invasive (MCF7 and T-47D) breast cancer cell lines. In highly invasive cell lines, PI(4)P levels in the Golgi apparatus were significantly higher than those in weakly invasive cell lines (Fig. 3C and D). Moreover, PI4KIIIβ expression was higher in late-stage human breast cancer tissues (metastatic, stages III and IV) than in early-stage tissues (nonmetastatic, stages I and IIa; Fig. 3E). In contrast, SAC1 expression decreased in stages III and IV human breast cancer tissues (metastatic; Fig. 3E). These data collectively support the idea that there is a strong correlation between Golgi PI(4)P and breast cancer malignancy.

PI4KIIIβ knockdown decreases PI(4)P levels in the Golgi apparatus and affects cell–cell adhesion and invasion

To further confirm the correlation between PI(4)P levels in the Golgi apparatus and cell–cell adhesion, we examined the effect of silencing PI4KIIIβ, which produces PI(4)P at the Golgi apparatus, in MCF7 cells. PI4KIIIβ knockdown decreased PI(4)P levels in the Golgi apparatus by half in MCF7 cells (Supplementary Fig. S3D–S3F). Because tight cell–cell adhesion already exists in MCF7 cells, further formation of cell–cell adhesion was not induced by PI4KIIIβ silencing in MCF7 cells (Supplementary Fig. S3B). We then used highly invasive MDA-MB-231 human breast adenocarcinoma cells that do not normally exhibit cell–cell adhesion and examined the 4 isoforms of PI4-kinase that might be involved in these phenotype changes. Of the four PI4-kinase isoforms examined, attenuation of only PI4KIIIβ promoted an increase in cell–cell adhesion in MDA-MB-231 cells (Supplementary Fig. S4A and S4B). Because PI4KIIIβ is known to produce PI(4)P in the Golgi apparatus, it may be involved in this change. In MDA-MB-231 cells, PI4KIIIβ knockdown decreased PI(4)P levels in the Golgi apparatus to one third of the control level (Fig. 4A and B).

MDA-MB-231 cells have been reported to utilize cadherin-11 rather than E-cadherin and N-cadherin as a cell-adhesion molecule (27, 28). PI4KIIIβ-depleted MDA-MB-231 cells formed adhesions with cadherin-11 and β-catenin without any changes in the expression levels of these proteins (Fig. 4C and D). In addition, further transfection of PI4KIIIβ-depleted MDA-MB-231 cells with Sac1 siRNA decreased their cell–cell adhesion (Supplementary Fig. S4C and S4D). Another line of highly invasive human ductal breast carcinoma cells, Hs578t cells, does not exhibit cell–cell adhesion under normal conditions. Depletion of PI4KIIIβ in these cells resulted in an increase in cell–cell adhesion (Supplementary Fig. S5A–S5C). Hs578t cells express N-cadherin and cadherin-11 but not E-cadherin (27, 28). Membrane localization of these cadherins was induced by PI4KIIIβ silencing without changes in protein levels (Supplementary Fig. S5A–S5C). MDA-MB-231 cell invasion was significantly suppressed by PI4KIIIβ depletion (Fig. 4E), with an increase in RhoA activity in these cells (Fig. 4F).

Furthermore, these cells exhibited decreased expression of SNAI1, an EMT marker (Fig. 4G). These data suggest that decrease in PI(4)P levels in the Golgi apparatus is likely to increase cell–cell adhesion, thereby inhibiting invasion and promotion of MET in breast cancer cells.

GOLPH3 is involved in PI(4)P-dependent changes in the phenotype

GOLPH3 is a downstream target of PI(4)P that is localized in the Golgi apparatus (9). In PI4KIIIβ-depleted MDA-MB-231 cells, GOLPH3 was no longer localized at the Golgi apparatus (Fig. 5A), without any significant change in its expression (Supplementary Fig. S6A). GOLPH3 expression did not change between control and SAC1 siRNA-transfected MCF7 cells (Supplementary Fig. S6B). GOLPH3 already localized at the Golgi apparatus in control siRNA-transfected MCF7 cells (Supplementary Fig. S6C); however, fluorescence intensity of GOLPH3 increased in SAC1-depleted MCF7 cells (Supplementary Fig. S6D). These data indicated that the PI(4)P in the Golgi apparatus regulates GOLPH3 localization but not expression. Because it has been reported that GOLPH3 is related to tumorigenesis (10, 15), we hypothesized that it may be involved in the regulation of cell–cell adhesion and cell migration/invasion. Attenuation of GOLPH3 resulted in increased cell–cell adhesion and suppression of the invasive phenotype, which was similar to what was observed during PI4KIIIβ knockdown (Fig. 5B–D). In addition, further GOLPH3 depletion in SAC1-depleted MCF7 cells decreased migration ability (Fig. 5E) and promoted cell–cell adhesion (Supplementary Fig. S6E). These data indicate that GOLPH3 is involved in cell–cell adhesion, as well as migration and invasion, which are dependent on Golgi PI(4)P.

PI(4)P-binding ability of GOLPH3 plays an important role in cancer progression

The GFP-tagged GOLPH3 R90L mutant (GFP-GOLPH3 R90L), which is unable to bind to PI(4)P (8, 9), also did not localize to the Golgi apparatus (Fig. 6A and Supplementary Fig. S6F). In MCF7 cells overexpressing GFP-GOLPH3, cell–cell adhesion decreased and was not altered by expression of the GFP-GOLPH3 R90L mutant (Fig. 6A). Cell migration was also promoted by overexpression of wild-type GOLPH3 in MCF7 cells but not by overexpression of the GOLPH3 R90L mutant (Fig. 6B). In MDA-MB-231 cells, expression of the GOLPH3 R90L mutant increased cell–cell adhesion but did not affect invasion (Fig. 6C and D), whereas expression of wild-type GOLPH3 abolished cell–cell adhesion and enhanced
Figure 6. Binding of PI(4)P to GOLPH3 is required for regulation of cell–cell adhesion and cell migration/invasion. A, GFP (mock), GFP-tagged wild-type GOLPH3 (GOLPH3 wt), or the GFP-tagged GOLPH3 R90L mutant (GOLPH3 R90L) was overexpressed in MCF7 cells. Cells were stained with an anti-E-cadherin antibody and phalloidin. Scale bar, 20 μm. B, mock, GOLPH3 wt, or GOLPH3 R90L-expressing MCF7 cells were used for a migration assay. The data shown are the mean (SEM) values of six independent experiments. (Continued on the following page.)
invasion (Fig. 6C and D). This enhanced invasion ability in GOLPH3-expressing cells was abolished by PHKIIβ depletion (Fig. 6E).

In addition, PI(4)P levels in the Golgi apparatus increased in GOLPH3 wt-expressing MCF-7 and MDA-MB-231 cells but not GOLPH3 R90L-expressing MCF-7 and MDA-MB-231 cells (Fig. 7A–D). However, in GOLPH3 wt-expressing MCF7 cells, upregulation of SNAI2 expression was not observed (Fig. 7E). In contrast, downregulation of SNAI2 expression was observed in GOLPH3 R90L-expressing MCF7 cells (Fig. 7E). This finding suggests that GOLPH3 is necessary but not sufficient for EMT induction. Finally, we tested the effect of GOLPH3 on cancer metastasis in vivo. GOLPH3 wt-expressing MDA-MB-231 cells showed much higher levels of metastasized to the lung than GFP-expressing cells (Fig. 7F and G). However, GOLPH3 R90L, which is a mutant with defective lipid binding, lost its metastasizing activity. These data indicate that GOLPH3 functions as a regulator of cancer metastasis in breast cancer through interaction with Golgi PI(4)P.

Discussion

In this study, we have shown that PI(4)P in the Golgi apparatus is a key regulator of cancer progression. In the Golgi apparatus, SAC1 and PI4KIIIβ are predominantly involved in PI(4)P metabolism. However, except for its role in the regulation of lipid homeostasis and sphingolipid biosynthesis, little is known about the specific role of SAC1-dependent dephosphorylation of PI(4)P (29–31). We showed that PI4KIIIβ and SAC1 expression and PI(4)P levels in the Golgi apparatus were correlated with cancer progression. According to the NextBio database (www.nextbio.com), SAC1 and PI4KIIIβ mRNA expression are correlated with many cancers, including breast cancer (Supplementary Tables S1 and S2). These results support our hypothesis that PI(4)P at the Golgi apparatus regulates cancer progression.

The Golgi apparatus is a central organelle, which is involved in protein and lipid biosynthesis and vesicular trafficking. Newly synthesized proteins and lipids are glycosylated in the Golgi apparatus. Although aberrant glycosylation and overexpression of glycolipids have been shown to be common features of malignant tumors (32, 33), the biologic significance of these changes is not well understood. PI(4)P is enriched at the Golgi apparatus, where PI(4)P-binding proteins that have been shown to be involved in vesicular transport, lipid transfer, and glycosylation are located (1). A Golgi PI(4)P effector, GOLPH3, is involved in Golgi-plasma membrane transport and in the glycosylation of proteins (8, 34, 35). In yeast, Vps74, the ortholog of human GOLPH3, binds directly to Sac1 (36). We found that expression of GOLPH3 wt, but not GOLPH3 R90L, resulted in an increase in PI(4)P levels in the Golgi apparatus. These data suggest that GOLPH3 overexpression competes with SAC1 for PI(4)P in the Golgi membrane, or that GOLPH3 senses PI(4)P levels at the Golgi apparatus, thus regulating SAC1 or PHKIIβ localization or activity. We observed that PI(4)P regulated GOLPH3 localization to the Golgi apparatus. These results suggest that GOLPH3 is localized to the Golgi apparatus by increasing PI(4)P, leading to aberrant glycosylation and overexpression of glycolipids.

EMT is an important process in cancer progression. It is characterized by a decrease in epithelial markers (e.g., E-cadherin) and upregulation of mesenchymal markers, including vimentin, SNAI1, and SNAI2, which together result in disruption of cell–cell adhesion. However, whether EMT/MET occurs in cancer progression remains controversial (37, 38). We observed that the membrane localization of cadherins and β-catenin and the expression of SNAI1 and SNAI2 are changed by PI(4)P generation at the Golgi apparatus, although decreases in the expression of E-cadherin, which is a characteristic of complete EMT, were not detected. Malignant cancer cells adopt some mesenchymal features but retain characteristics of epithelial cells (39–41), a condition that has been described as incomplete EMT (42). Taken together with the finding that the amount of PI(4)P increases at the Golgi apparatus during EMT, our data suggest that Golgi PI(4)P is necessary but not sufficient for EMT.

Several studies have shown that GOLPH3 plays important roles in the progression of several tumor types. For example, GOLPH3 upregulated and promotes proliferation and tumorigenicity in breast cancer (15). Recent studies have demonstrated the clinical significance of GOLPH3 in several cancers (13, 14, 43). A very recent study reported that GOLPH3 contributes to the migration and invasion of glioma cells through RhoA expression (44). We found that changes in PI(4)P levels at the Golgi apparatus resulted in alteration of RhoA activity. RhoA activity is required for initial cell–cell adhesion formation (45) and is repressed during EMT (45). Taken together with our results, these findings suggest that Golgi PI(4)P and GOLPH3 regulate the cytoskeletal rearrangement of actin through RhoA as well as Golgi-plasma membrane transport during tumor progression.

In conclusion, our data suggest that PI(4)P levels in the Golgi apparatus play a critical role in cancer progression by regulating the PI(4)P effector GOLPH3 (Supplementary Fig. S6G). In this study, we showed that changes in Golgi PI(4)P levels and/or Golgi localization of GOLPH3 were likely to result in changes in cell–cell adhesion and cell migration/invasion in breast cancer cell lines. Furthermore, we showed that PI(4)P-

(Continued.) * P < 0.0005. Scale bar, 100 μm. C, mock, GOLPH3 wt, or GOLPH3 R90L-expressing MDA-MB-231 cells were stained with an anti–β-catenin antibody and phalloidin. Scale bar, 20 μm. D, mock, GOLPH3 wt, or GOLPH3 R90L mutant-expressing MDA-MB-231 cells were seeded onto Matrigel invasion chambers. Eighteen hours after seeding, the cells were fixed and stained with phalloidin. The number of invasive cells that passed through the Matrigel chambers was counted. The data shown are the mean (SEM) values of four independent experiments. ** P < 0.005; *** P < 0.0005. Scale bar, 100 μm. E, mock or GOLPH3 wt-expressing MDA-MB-231 cells were transfected with the indicated siRNAs. Forty-eight hours after transfection, the cells were serum starved and seeded onto Matrigel invasion chambers. Eighteen hours after seeding, the cells were fixed and stained with phalloidin. The number of invasive cells that passed through the Matrigel chambers was counted. The data shown are the mean (SEM) of four independent experiments. **** P < 0.0005. Scale bar, 100 μm.
Figure 7. Binding of PI(4)P to GOLPH3 is required for metastasis in vivo. A, GFP-, GOLPH3 wt-, or GOLPH3 R90L-expressing MCF7 cells were transfected with the indicated siRNAs and then fixed and stained with anti-GM130 and the Alexa Fluor 647–labeled Fapp1 2 × PH domain. Scale bar, 20 μm. B, Golgi PI(4)P levels were quantified in GFP-, GOLPH3 wt-, or GOLPH3 R90L-expressing MCF7 cells. Significant increases were observed following GOLPH3 wt expression. The data shown are the mean (SEM) values from more than 100 cells. *, P < 0.001. C, GFP-, GOLPH3 wt-, or GOLPH3 R90L-expressing MDA-MB-231 cells were transfected with the indicated siRNAs and then fixed and stained with anti-GM130 antibody and the Alexa Fluor 647–labeled Fapp1 2 × PH domain. Scale bar, 20 μm. D, Golgi PI(4)P levels were quantified in GFP-, GOLPH3 wt-, or GOLPH3 R90L-expressing MDA-MB-231 cells. The data shown are the mean (SEM) values from more than 100 cells. *, P < 0.001. E, total RNA was isolated from GFP- or GFP-tagged GOLPH3 stable MCF7 cells. The SNAI2 mRNA level was quantified by real-time PCR. The data shown are the mean (SEM) values of three independent experiments. *, P < 0.0001. F, lung surface metastatic foci were counted. The data shown are the mean (SEM) values from seven (mock and GOLPH3 wt) or 8 (GOLPH3 R90L) mice. *, P < 0.04; **, P < 0.02. G, representative images of the lungs with metastatic foci.
binding ability of GOLPH3 was essential for metastasis in vivo. Therefore, molecules that modulate the PI(4)P level in the Golgi apparatus, such as SAC1 and PI4KIIIβ, may represent intriguing anticancer therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E. Tokuda, T. Itoh, T. Takenawa

Development of methodology: E. Tokuda, T. Takenawa

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Tokuda, T. Takenawa, Y. Irino, M. Fukimoto

Analysis and interpretation of data (e.g., statistical analysis, biosistatistics, computational analysis): E. Tokuda

Writing, review, and/or revision of the manuscript: E. Tokuda, T. Itoh, J. Haugen, T. Takenawa

Study supervision: T. Itoh, T. Takenawa

References


Acknowledgments

The authors thank T. Oikawa (Department of Molecular Biology, Hokkaido University Graduate School of Medicine) for the discussions and Y. Murata and T. Yanagita (Division of Molecular and Cellular Signaling, Kobe University Graduate School of Medicine) for the technical advice.

Grant Support

This study was supported by a grant-in-aid for Scientific Research (S) (grant number 23227005 to T. Takenawa) and a grant-in-aid for Challenging Exploratory Research (grant number 24506211 to E. Tokuda) from Japan Society for the Promotion of Science.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 23, 2013; revised February 14, 2014; accepted March 3, 2014; published OnlineFirst April 4, 2014.
Phosphatidylinositol 4-Phosphate in the Golgi Apparatus Regulates Cell –Cell Adhesion and Invasive Cell Migration in Human Breast Cancer

Emi Tokuda, Toshiki Itoh, Junya Hasegawa, et al.

*Cancer Res* 2014;74:3054-3066. Published OnlineFirst April 4, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-2441

Supplementary Material  Access the most recent supplemental material at: [http://cancerres.aacrjournals.org/content/suppl/2014/04/04/0008-5472.CAN-13-2441.DC1](http://cancerres.aacrjournals.org/content/suppl/2014/04/04/0008-5472.CAN-13-2441.DC1)

Cited articles  This article cites 45 articles, 15 of which you can access for free at: [http://cancerres.aacrjournals.org/content/74/11/3054.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/74/11/3054.full.html#ref-list-1)

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at: [http://cancerres.aacrjournals.org/content/74/11/3054.full.html#related-urls](http://cancerres.aacrjournals.org/content/74/11/3054.full.html#related-urls)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.