Phosphatidylinositol 3–Kinase Is Required for the Formation of Constitutive Transport Vesicles from the TGN

Steven M. Jones and Kathryn E. Howell
Department of Cellular and Structural Biology, University of Colorado School of Medicine, Denver, Colorado 80262

Abstract. An 85-kD cytosolic complex (p62<sup>plx</sup>), consisting of a 62-kD phosphoprotein (p62) and a 25-kD GTPase, has been shown to be essential for the cell-free reconstitution of polymeric IgA receptor (pIgA-R)-containing exocytic transport vesicle formation from the TGN (Jones, S.M., J.R. Crosby, J. Salamero, and K.E. Howell. 1993. J. Cell Biol. 122:775–788). Here the p62<sup>plx</sup> is identified as a regulatory subunit of a novel phosphatidylinositol 3–kinase (PI3-kinase). This p62<sup>plx</sup>-associated PI3-kinase activity is stimulated by activation of the p62<sup>plx</sup>-associated GTPase, and is specific for phosphatidylinositol (PI) as substrate, and is sensitive to wortmannin at micromolar concentrations. The direct role of this p62<sup>plx</sup>-associated PI3-kinase activity in TGN-derived vesicle formation is indicated by the finding that both lipid kinase activity and the formation of pIgA-R–containing exocytic vesicles from the TGN are inhibited by wortmannin with similar dose-response curves and 50% inhibitory concentrations (3.5 μM). These findings indicate that phosphatidylinositol-3-phosphate (PI[3]P) is required for the formation of TGN-derived exocytic transport vesicles, and that the p62<sup>plx</sup>-associated PI3-kinase and an activated GTPase are the essential molecules that drive production of this PI[3]P.
phorylation/dephosphorylation cycle that controls p62p62px association with the TGN membrane, raises the question of how the p62p62px acts to stimulate vesicle formation. To address this question, we have undertaken a biochemical characterization of the function of the p62p62px.

In this paper, the p62 molecule is shown to share primary sequence homology with the p85α regulatory subunit of a PI3-kinase and, consistent with the homology data, the membrane-associated p62p62px regulates phosphatidylinositol (PI)-specific, and wortmannin-inhibitable PI3-kinase activity. From these data we conclude that a critical pool of phosphatidylinositol-3-phosphate (PI3P) is required for the formation of TGN-derived exocytic transport vesicles, and that the p62p62px-associated PI3-kinase and an activated GTPase are the essential molecules that drive production of this PI(3)P pool.

Materials and Methods

Chemical Reagents

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Indianapolis, IN). 10 mM wortmannin in DMSO was stored in aliquots at −70°C and each aliquot was used only once. LysC was purchased from Waco Chemicals (Waco, TX). Phosphatidylinositol was purchased from Avanti Polar Lipids (Alabaster, AL).

Antibodies

Production of specific antibodies against p62, TGN38, and the p85A-R has been described (Sztul et al., 1985; Luzio et al., 1990; Jones et al., 1993). p62p62px antibodies (rabbit 950) were raised against the 85-kD cytosolic complex, and were isolated using immunoaffinity chromatography. Monoclonal antibodies against TGN38 were from hybridoma 2F7.1, provided by G. Banting (University of Bristol, Bristol, U.K.) (Horn and Banting, 1994). Preimmune sera used in all experiments is from rabbit 950. Antibodies against p85α and p110αβ were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against wortmannin were the gift of Dr. R. Abrahams (The Mayo Foundation, Rochester, MN) (Brunner et al., 1996).

Subcellular Fractionation Procedures

Stacked Golgi fractions (SGFs) were isolated from rat liver using modifications of the method of Lelavathi et al. (1970) (Taylor et al., 1997b). Livers were removed, finely minced and resuspended at 6 g per 10 ml 0.5 M sucrose in 100 mM KPO4, pH 6.8, 5 mM MgCl2, and 1 μg/ml each of a mixture of proteolytic inhibitors: chymostatin, leupeptin, antipain, and pepstatin. All sucrose solutions contained the same buffer and proteolytic mixture of proteolytic inhibitors: chymostatin, leupeptin, antipain, and pepstatin. The adjusted SII was loaded into the bottom of an Islanders (Lexington, KY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against wortmannin were the gift of Dr. R. Abrahams (The Mayo Foundation, Rochester, MN) (Brunner et al., 1996).

Immunoprecipitates or isolated complexes (5 μl) was added to the samples and they were rotated at 4°C for 2 h. Immunoprecipitates were pelleted for 1 h at 4°C with sheep antibodies against the Fc domain of rabbit IgG, which were covalently coupled to fibrous cellulose (Luzio, 1977). Immune complexes were pelleted and washed seven times: once with E buffer; twice with RIPA (10 mM Na phosphate, pH 7.0, 1% NP-40, 1% Na deoxycholate, 0.1% SDS, 2 mM EDTA, 20 mM NaF, 2 mM Na vanadate), twice with PAN–NP–40 (20 mM Pipes, pH 7.4, 100 mM NaCl, 1% NP-40), and then twice with PAN (the proteolytic inhibitors were included in all washing buffers). The immune precipitates were resuspended in 20 μl PAN and frozen in 5 μl aliquots for PI3-kinase assays.

PI3-kinase Assays

PI3-kinase assays were as described by Kazlauskas and Cooper (1999). Immune-precipitates or isolated complexes (5 μl) in PAN, were resuspended in a reaction mixture containing 20 mM Hepes, pH 7.4, 5 mM MgCl2, 0.45 mM EGTA, 10 μM ATP (~5 μCi [γ32P]ATP) and 0.2 mg/ml phosphatidylinositol (PI) in a final reaction volume of 10 μl and then incubated 0–20 min at 30°C. After incubation, the reaction was stopped with 100 μl 1 M HCl and the lipids extracted with 200 μl CHCl3/MeOH (1:1).

Phosphorylation/Dephosphorylation Cycle

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followed by 80 μl 1 M HCl/MeOH (1:1) and dried in a speed vac (Savant Instruments Inc., Holbrook, NY). The samples were resuspended in 10 μl CHCl/MeOH (1:1) and spotted onto Silica Gel 60 TLC plates (JT Baker Chromatography, Union City, CA). The TLC plates were pretreated with 60 mM EDTA, 2% Na tartrate, and 50% EtOH, and then were dried in a 100°C oven overnight. Development of the TLC plates was in CHCl/MeOH/4 N NH₄OH (9:7:2) for ~2 h. The TLC plates were dried and exposed to film for autoradiography and phosphorimager analysis. Positive and negative controls were immunoprecipitates of stimulated and non-stimulated PDGF receptors, provided by A. Kazlauskas (Schepps Eye Institute, Boston, MA).

**Gel Filtration Chromatography**

Gel filtration chromatography was carried out using Sephacryl S500 (Pharmacia Fine Chemicals). Native SGF (2.0 mg, not high pH washed) was solubilized in CHAPS buffer, loaded onto the column, and eluted with 20 mM Hepes-KOH, pH 6.8, 100 mM KCl, 0.3 M sucrose, 20 mM CHAPS containing the cocktail of proteolytic inhibitors. 3.0 ml fractions were collected, and half were immunoprecipitated for PI3-kinase assays and the other half TCA precipitated for SDS-PAGE and immunoblot analysis. The column was calibrated with molecular weight standards: 660-kD thyroglobulin; 443-kD apoferritin; 232-kD β-amylase; and 66-kD BSA.

**Cell-free Assay of pIgA-R–Containing Exocytic Vesicle Formation from the TGN**

The cell-free assay of budding from an immobilized SGF was carried out as described by Salamero et al. (1990) and Howell et al. (1994). Each assay contains 2.5 mg magnetic core and shell beads with ~50 μg SGF immobilized. The immobilized fraction is characterized in Jones et al. (1997). For the budding reaction the immobilized fraction was incubated in 2.5 ml, containing 0.70 mg/ml cystosol, 25 mM Hepes, pH 6.7, 25 mM KCl, 1.5 mM Mg acetate, 1.0 mM ATP, an ATP regenerating system (8.0 mM creatine phosphate, 0.043 μM/ml creatine phosphokinase, and 5 mg/ml BSA [final concentrations]). After 10 min at 37°C, the Golgi fraction remaining on the beads was retrieved with a magnet and the budded vesicles remained in the supernatant. The budded fraction was pelleted through a 0.25 M sucrose cushion (for 1 h at 100,000 g), to reduce the large amounts of cytosolic protein and 5 mg/ml BSA present in the budding reaction. The high concentration of soluble protein made it impractical to carry out gel analysis on the total budded fraction. The pellet was resuspended in gel sample solvent and 5 mg/ml BSA present in the budding reaction. The high concentration of soluble protein made it impractical to carry out gel analysis on the total budded fraction. The pellet was resuspended in gel sample solvent and 5 mg/ml BSA present in the budding reaction. The high concentration of soluble protein made it impractical to carry out gel analysis on the total budded fraction. The pellet was resuspended in gel sample solvent and 5 mg/ml BSA present in the budding reaction. The high concentration of soluble protein made it impractical to carry out gel analysis on the total budded fraction. The pellet was resuspended in gel sample solvent and 5 mg/ml BSA present in the budding reaction. The high concentration of soluble protein made it impractical to carry out gel analysis on the total budded fraction. 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These data suggest that p62 may represent a novel regulatory subunit of a PI3-kinase that shares some identity with p85α. Other possibilities are that it might be a shorter form of a p85 regulatory subunit, a proteolytic fragment of p85α, or a protein that shares a common bcr domain (or GTPase binding domain) with p85.

**p62 Immunoprecipitates Have PI3-kinase Activity**

Enzymatic assays were carried out to directly determine whether PI3-kinase activity is associated with the p62<sup>ph</sup>. Since the assay for PI3-kinase associated with PDGF receptors was established using immunoprecipitates of the activated receptor (Kazlauskas and Cooper, 1990), we used immunoprecipitates of activated and nonactivated PDGF receptor as controls. Activated PDGF receptors generate PI(3)P from PI, therefore, the mobility of the PI(3)P serves as a standard on the TLC plates. For the assay, immunoprecipitates were prepared from CHAPS-solubilized native SGF using antibodies raised against characterized components of the membrane-associated p62<sup>ph</sup>: TGN38 (luminal domain, polyclonal); p62; p62<sup>ph</sup>; and TGN38 (luminal domain, monoclonal). As shown in Fig. 1, A and B, PI3-kinase activity was readily detected in all immunoprecipitates containing the membrane-associated p62<sup>ph</sup>. The level of activity measured was at least as robust as that obtained for the unstimulated PDGF receptor. This comparison is germane because the p62<sup>ph</sup> activity most likely represents the unstimulated endogenous activity. Controls for the specificity of the assays included immunoprecipitates derived from the same fraction using either preimmune sera or antibodies directed against the p15A-R, an abundant transmembrane protein of SGF. The control immunoprecipitates had minimal kinase activity. The amount of p62<sup>ph</sup>-associated PI3-kinase activity obtained with the immunoprecipitates correlated with the amount of p62 precipitated by the different antibodies.

| Subunit | Sequence Comparison of p62 |
|---------|---------------------------|
| p62, peptide1 | XXISPFXKPQPPRPPLPVAPGS | 21 |
| p62, peptide2 | XXISPFXKPQPPRPPLPVAPGSSK | 24 |
| p85_MOUSE | XXISPFXKPQPPRPPLPVAPGSSK | 105 |
| p85_HUMAN | XXISPFXKPQPPRPPLPVAPGSSK | 105 |
| p85_BOVIN | XXISPFXKPQPPRPPLPVAPGSSK | 105 |

Table I. p62 Has Sequence Identity to the p85 Regulatory Subunits of PI3-kinases

Alignment of two different peptides generated from p62 (21 and 24–amino acids long) with amino acids 82–105 of the p85α subunit of PI3-kinases from mouse, human, and bovine. Asterisks at the bottom of the sequence indicate identical amino acids between p62-peptide2 and the p85α subunits. Noted in bold in the p62-peptide sequences is a threonine residue in p62-peptide2 which could not be determined in p62-peptide1.
GTPases have been demonstrated to associate with and stimulate PI3-kinases (Zhang et al., 1993; Kodaki et al., 1994; Zheng et al., 1994; Tolias et al., 1995; Bokoch et al., 1997), in vitro phosphorylation reactions performed in an attempt to identify the p62<sup>pix</sup>-associated PI3-kinase catalytic subunit. The p62<sup>pix</sup> was purified from detergent-solubilized, high pH-treated, membrane-associated p62<sup>pix</sup> recovered from native SGF (without carbonate treatment) as labeled on top of each panel. Antibodies used for the immunoprecipitates are labeled at the bottom of each panel. The origin and mobility of PI(3)P is labeled on the right of D.

Phosphorylation Identifies a 100-kD Protein in the p62<sup>pix</sup> Purified from Detergent-solubilized Native SGF

Since in the yeast system the PI3-kinase regulatory subunit (Vps 15p) is a protein kinase that has been postulated to phosphorylate the PI3-kinase catalytic subunit (Vps34p) (Stack et al., 1995a) and it has been shown that the mammalian p110<sup>y</sup> subunit can autophosphorylate (Vanhaesebroeck et al., 1997), in vitro phosphorylation reactions were performed in an attempt to identify the p62<sup>pix</sup>-associated PI3-kinase catalytic subunit. The p62<sup>pix</sup> was purified from detergent-solubilized, high pH washed, and native membranes using an immunoaffinity column prepared by covalently attaching an IgG fraction of p62 antisera. Kinase assays demonstrated that a single band at 62 kD was phosphorylated when the p62<sup>pix</sup> was purified from detergent-solubilized, high pH–washed SGF and the phosphor-
we conclude that the membrane-associated p62cplx contains file as the p62 subunit (Fig. 3, of this subunit exhibited the same calcium activation pro-

an additional 100-kD subunit that is detected only when detergent-solubilized, high pH–washed SGF (Fig. 3) (10 ng) was incubated in an in vitro phosphorylation re-

The mobility of p62 is noted at the left of A and B. Quantitation of the phosphorylation is plotted in PhosphorImager units (C).

did not show), an additional ~100-kD phos-

Wortmannin Binding Identifies a 100-kD Protein in the p62phosphorylated subunit was present and the phosphorylation of this subunit exhibited the same calcium activation profile as the p62 subunit (Fig. 3, B and C). From these data we conclude that the membrane-associated p62phlx contains an additional 100-kD subunit that is detected only when the SGF is not high pH washed before detergent solubilization. Both the 62- and 100-kD subunits are likely to be phosphorylated by the same kinase because their phosphorylation displays the same calcium activation curve.

**Wortmannin Binding Identifies a 100-kD Protein in the p62phlx Purified from Detergent-solubilized Native SGF as the PI3-kinase Catalytic Subunit**

Wortmannin inhibits PI3-kinases by covalently coupling to the active site of the catalytic subunit and this interaction is stable after resolution by SDS-PAGE (Wymann et al., 1996). The bound wortmannin can be identified by using either [3H]wortmannin and autoradiography or an antibody against wortmannin and immunoblotting. Brunn et al. (1996) (using rat brain extracts and in vitro incubations) showed wortmannin covalently coupled to a very broad band of proteins that span 100–110 kD, as well as several proteins at ~200 kD. To identify the catalytic subunit associated with the membrane-bound, p62phlx-associated PI3-kinase, fractions were incubated with wortmannin, resolved by SDS-PAGE, and then immunoblotted with antibodies against wortmannin. In rat liver, cytosol bands of 100, ~130, and ~200 kD bind wortmannin. Immunoprecipitates of the membrane-bound, p62phlx-associated PI3-kinase (the same as those used for the PI3-kinase assays) contains only the 100-kD wortmannin binding band and this band is not precipitated by the preimmune sera (Fig. 4). The findings that the immunoprecipitates have PI3-kinase activity and a 100-kD wortmannin binding protein, support the identification of the 100-kD band as the PI3-kinase catalytic subunit.

**Neither the 62- Nor the 100-kD Subunits Are Antigenically Related to p85α or p110α and p110β**

The antigenic relationship of p62 and p85α and the 100-kD subunit and p110α and p110β was tested using available antibodies. Immunoblot analysis was carried out on rat liver fractions: PNS, cytosol (Cyt), SGF, and the p62cplx purified from detergent-solubilized, high pH–washed SGF (complex contains in addition to p62, the 25-kD GTPase, TGN38, and the 100-kD wortmannin-binding subunit). Antibodies directed against the p85α, p110α, and p110β subunits of the growth factor–

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Figure 3. Phosphorylation of p62phlx purified from detergent-solubilized native SGF reveals a 100-kD subunit not present if the SGF is high pH–washed before solubilization, p62phlx purified from detergent-solubilized, high pH–washed SGF (A) and native SGF (B) (10 ng) was incubated in an in vitro phosphorylation re-

Figure 4. Wortmannin binding identified a 100-kD protein in the p62phlx as the PI3-kinase catalytic subunit. Total cytosol (100 μg, cytosol) or immunoprecipi-

neither 62-kD subunit, nor the 100-kD subunit, nor the 100-kD subunit is antigenically related to p85α or p110α and p110β.

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Little or no reactivity with these antibodies was observed in SGF and p62phlx fractions. Using antibodies directed against p62, an immunoreactive 62-kD band was detected in all of the fractions (albeit only weakly in PNS), and the electrophoretic mobility of both p62 and p85 bands were clearly differentiated in these same fractions. The demonstration that p62 exhibited a consistent molecular mass in all fractions tested, including PNS, suggested that p62 was unlikely to represent a proteolytic fragment of p85α. Antibodies directed against TGN38 confirmed the presence of this transmembrane protein in PNS, SGF, and the p62phlx isolated from membranes. However, we were unable to immunologically con-
firm the presence of a known PI3-kinase catalytic subunit in the p62 complex as none of the available monoclonal antibodies were able to recognize any subunit of the immunopurified membrane form of p62<sup>plk</sup>.

**Identification of PI3-kinase Activity in p62<sup>plk</sup> Purified from Detergent Extracts of Native SGF**

Sephacryl S500 gel filtration chromatography of CHAPS-solubilized native SGF was used to identify a membrane-bound, p62<sup>plk</sup>-associated PI3-kinase activity (Fig 6). Two major peaks of TGN38 and p62 immunoreactive materials were eluted at ~250 and ~700 kD. Only immunoprecipitates of p62 from the ~700-kD peak contained PI3-kinase activity. These data indicate that a PI3-kinase catalytic subunit (100-kD protein) associates with TGN38 and p62 to form a significantly larger complex than the p62<sup>plk</sup>/TGN38-containing complex recovered from high pH-treated Golgi membranes, and that this larger complex contains PI3-kinase activity.

**Properties of the p62<sup>plk</sup>-associated PI3-kinase Activity**

The biochemical properties of the p62<sup>plk</sup>-associated PI3-kinase activity were determined and compared with those of several other PI3-kinases, including the growth factor–associated p110, Vps34p, and the human Vps34p homologue (Stack et al., 1995a; Volinia et al., 1995) (Figs. 7 and 8; and Table II). The 50% inhibitory concentration (IC<sub>50</sub>) of the p62<sup>plk</sup>-associated PI3-kinase activity with wortmannin was ~3.5 μM. This value is similar to that determined for Vps34p (~3 μM) and is three orders of magnitude greater than the IC<sub>50</sub> of the p110 PI3-kinase and of the mammalian Vps34p homologue (2–3 NM). The p62<sup>plk</sup>-associated PI3-kinase activity was also stimulated by low concentrations of non-ionic detergent (0.1% NP-40), similar to p110 and Vps34p, whereas all these PI3-kinases were inhibited by NP-40 at higher concentrations. p62<sup>plk</sup>-associated PI3-kinase and all other PI3-kinase activities were insensitive to high (nM) concentrations of adenosine (data not shown). These properties are used to distinguish PI3-kinases (activation by low concentration of detergent and insensitivity to adenosine) from phosphatidylinositol 4–kinases, which are not detergent activated and are sensitive to adenosine.

The substrate specificity of the p62<sup>plk</sup>-associated activity was assayed, in the absence or presence Mg<sup>2+</sup> or Mn<sup>2+</sup>, with P1, phosphatidylinositol-4-phosphate (P1[4]P), phosphatidylinositol-4,5-diphosphate (P1[4,5]P2) as substrates. The p62<sup>plk</sup>-associated activity showed a preference for Mn<sup>2+</sup> and a specificity for P1 (Fig. 7). Although the full set of data on the properties of each PI3-kinase is not available, the biochemical properties of the p62<sup>plk</sup>-associated PI3-kinase were most similar to those of Vps34p. In summary, the lipid kinase activity associated with the p62<sup>plk</sup> is a PI-specific, Mn<sup>2+</sup>-activated PI3-kinase that is sensitive to wortmannin at micromolar concentrations.

**Correlation between the Inhibition of p62–PI3-kinase Activity and the Formation of Exocytic Vesicles In Vitro**

The p62<sup>plk</sup> has been shown to be essential for cell-free formation of plgA-R–containing exocytic vesicles from the TGN (Jones et al., 1993). In this cell-free assay, an SGF is immobilized on magnetic beads, and in the presence of cytosol, ATP, and an ATP regenerating system at 37°C a mixed population of vesicles bud (Salamero et al., 1990). The amount of the mature sialylated plgA-R (116 kD) present in the budded fraction is quantitated and compared with that present in the starting Golgi fraction to determine budding efficiency of this population of vesicles.

If PI3-kinase activity is essential for vesicle formation, then the vesicle budding reaction should be wortmannin inhibitable, and the pharmacology of this inhibition should closely resemble that of the p62<sup>plk</sup>-associated PI3-kinase activity. The data shown in Fig. 8, A and B were consistent with both predictions. Increasing concentrations of wortmannin inhibited both the formation of plgA-R–containing vesicles and the PI3-kinase activity with similar dose-response curves and IC<sub>50</sub> of ~3–5 μM.
Discussion

The cytosolic p62<sub>cyto</sub> is required for the formation of pIgA-R-containing exocytic vesicles from the TGN in a cell-free assay system (Jones et al., 1993). A biochemical characterization of this complex is presented here and we discuss the function of the p62<sub>cyto</sub> in the process of vesicle formation from the TGN.

p62<sup>cyto</sup> Regulates a Novel PI3-kinase Activity

Sequence analysis of peptides derived from the p62 subunit of the p62<sup>cyto</sup> revealed a region of homology within this protein to the regulatory subunit, p85<sub>a</sub> of a PI3-kinase (Table I). The 24-amino acid sequence of p62 was identical to amino acids 82–105 in the brc domain of mouse p85<sub>a</sub>. No other homology with any other protein was found indicating that the homology was not to a generalized domain that binds small GTPases and found in many proteins. The stronger data supporting that p62 is a regulatory subunit of a PI3-kinase comes from the biochemical characterization. Enzyme assays of immunopurified p62<sup>cyto</sup> from an SGF confirmed p62 as a regulatory subunit of a PI3-kinase (Figs. 1–3, and 6–8). Maximal production of PI(3)P by the p62<sup>cyto</sup>-associated PI3-kinase activity showed a substrate specificity for PI, and an IC<sub>50</sub> for wortmannin of 3.5 μM (Table II, and Fig. 8). Inhibition of the formation of the pIgA-R-containing exocytic vesicles in vitro, by wortmannin had the same dose-response curve as the p62<sup>cyto</sup>-associated PI3-kinase activity (Fig. 8). These data implicate a role for PI(3)P in the multitude of steps that are required to sort molecules and form vesicles at the TGN.
in vitro, but their in vivo substrate is PI(4,5)P₂, and these exhibit an IC₅₀ for wortmannin in the low nM range (for reviews see Panayotou et al., 1993; Liscovitch and Cantley, 1994, 1995; Carpenter and Cantley, 1996). The regulatory (p85) and catalytic (p110) subunits of these PI3-kinases are associated with each other in cytosol and the p85 subunits are not protein kinases. Upon ligand binding to (and activation of) a receptor tyrosine kinase, the activated growth factor receptor–associated PI3-kinases bind phosphorylated tyrosines in the receptor cytoplasmic domain via an SH2 domain in the regulatory subunit. Thus, the PI3-kinase assembles en bloc with the activated receptor (for review see Kazlauskas, 1994). The TGN membrane receptor for the p62cplx-associated PI3-kinase is dimeric TGN38, but the interaction motif remains undefined. It probably does not involve a phosphotyrosine signal. The sole tyrosine in the cytoplasmic domain of TGN38 does

Figure 7. Properties of the p62cplx-associated PI3-kinase. PI3-kinase assays were carried out as described in Materials and Methods. Either Mg²⁺ (A) or Mn²⁺ (B) were used as the cation in the presence of multiple substrates: PI, PI(4)P, and PI(4,5)P₂, as listed at the top of each lane. The mobility of PI(3)P is noted at the right of B.

Figure 8. The p62cplx-associated PI3-kinase activity and the formation of pIgA-R–containing vesicles from the TGN are sensitive to the same concentrations of wortmannin. PI3-kinase assays were carried out as described in Materials and Methods at increasing concentrations of wortmannin (0.001–10 μM). The amount of PI(3)P formed was determined by PhosphorImager quantitation of the TLC plates. The activity expressed as percent of control (no wortmannin) is plotted versus wortmannin concentration (A). The cell-free assay was carried out as described in Materials and Methods at increasing concentrations of wortmannin (0.01–10 μM). The efficiency of formation of pIgA-R–containing vesicles was determined and is plotted versus wortmannin concentration (B).

Only the membrane-associated p62cplx isolated from non-high pH–treated SGF had PI3-kinase activity, and an additional 100-kD phosphoprotein subunit was detected in the active complex. The phosphorylation of p62 and p100 followed the same Ca²⁺ activation profile, suggesting that both subunits are phosphorylated by the same protein kinase (Fig. 3). Although it cannot be ruled out that the p100 subunit can autophosphorylate, as has been shown for p110γ (Vanhaesebroeck et al., 1997). Since the immunopurified p62cplx from detergent-solubilized, high pH–washed SGF (containing TGN38, p62, and a 25-kD GTPase) is shown to have protein kinase activity, p62 itself is likely to be the protein kinase. However, the possibility that the immunopurified material is contaminated with a small amount of a protein kinase cannot be ruled out. Confirmation of the protein kinase activity of p62 will require cloning and sequencing of the molecule. Importantly, a 100-kD wortmannin binding protein was identified in the enzymatically active p62cplx, identifying the p100 as the catalytic subunit of p62cplx-associated PI3-kinase. A number of genes encoding PI3-kinase catalytic subunit isoforms have been cloned, and the products found to be ~100 kD, therefore this data does not identify the specific isoform associated with the p62cplx (for review see Liscovitch and Cantley, 1994). Consistent with the biochemical data, the potential relationship of p100 as a substrate for the p62 protein kinase recapitulates the predicted situation in yeast vacuolar protein sorting where the Vps15p serine/threonine kinase is thought to phosphorylate the associated Vps34p catalytic subunit of PI3-kinase (Stack and Emr, 1994; Stack et al., 1995a).

The molecular interactions between components of p62cplx and its associated PI3-kinase catalytic subunit are quite stable as evidenced by having withstood the multiple detergents used in the immunoprecipitations. In gel filtration experiments, the enzymatically active complex resolved with an apparent molecular size of 700 kD. Since the p62cplx isolated from high pH–treated Golgi membranes was 250 kD (Jones et al., 1993), and the catalytic subunit is 100 kD, the molecular mass of the complex suggests either that the complex is dimeric or other signaling molecules are associated with the isolated complex.

The properties of the p62cplx-associated PI3-kinase differ from those of the activated growth factor receptor–associated PI3-kinases. The latter PI3-kinases phosphorylate PI
not reside in a proper context for phosphorylation, nor is it phosphorylated under the conditions we have used to study the reversible phosphorylation of p62.

The p62<sup>phk</sup>-associated PI3-kinase activity is more comparable biochemically to the trafficking PI3-kinase, Vps15p/Vps34p, characterized in *Saccharomyces cerevisiae* (for review see Stack et al., 1995b). Both are PI-specific PI3-kinases with IC<sub>50</sub> for wortmannin of ~3 μM. In addition, Vps15p-protein kinase is required to recruit Vps34p to the membrane and activate the production of PI(3)P (Stack et al., 1995a). The behavior of p62 is consistent with the Vps15p/Vps34p paradigm in that p62 is likely to be the kinase that phosphorylates the 100-kD protein, a wortmannin-binding protein, and presumably the catalytic subunit of the p62<sup>phk</sup>-associated PI3-kinase. However, we have no direct evidence that this phosphorylation results in activation of the PI3-kinase leading to increased production of PI(3)P.

Recently it has been proposed that PI3-kinase can be divided into three different classes (Domin and Waterfield, 1997). The first class is made up of the classical signaling PI3-kinase, which includes the components of the p85/p110 heterodimeric complexes. These lipid kinases have substrate specificity in vivo for PI(3,4,5)P<sub>3</sub> and are stimu-

| Potential Effectors of PI(3)P in Membrane Traffic |
|-----------------------------------------------|
| In vivo levels of PI(3)P in mammalian cells are relatively low, reasonably constant, and do not change upon growth factor stimulation (De Camilli et al., 1996; Shpetner et al., 1996). These data suggest that in signaling PI(3)P acts locally, and through interaction with different effectors, regulates different and even diverse functions. |
| One hypothesis for the role of PI(3)P in vesicle budding is that its presence in the outer leaflet of the bilayer drives outward curvature of the membrane, thereby facilitating bud formation (Stack et al., 1995b). This hypothesis is a direct derivation of the bilayer-couple model of Sheetz and Singer (1974) that relates the headgroup size and charge of phospholipids exhibiting significant local bilayer asymmetries with membrane curvature. Other hypotheses that account for the rather constant amounts of PI(3)P in cells are equally tenable at this time. The PI(3)P may be bound to a carrier molecule, like phosphatidyllinositol transfer protein, rather than being incorporated into the lipid bilayer (Bankaitis et al., 1990). This scenario would implies PI(3)P as a second messenger. |
| Many phosphatidylinositol and phosphoinositide-sensitive proteins with diverse functions have been identified: (a) isoforms of protein kinase C (Singh et al., 1993; Toker et al., 1994; Akimoto et al., 1996); (b) dynamin GTPase activity (Tuma, 1993); (c) calcium channels (Nori et al., 1993); (d) actin binding proteins (Weeds and Maciver, 1993; Sohn and Goldschmidt-Clermont, 1994); and (e) unconventional, nontransmembrane channels formed by vesicle coat proteins (e.g., coatamer and the AP-2 and AP-3 clathrin adapter complexes) (Timerman et al., 1990; Kijima et al., 1993; Fleisher et al., 1994). All of these PI(3,4,5)P<sub>3</sub>-sensi- |
tive molecules play roles in membrane traffic, albeit diverse roles. It now remains for us to identify the PI(3)P effector molecule/s that regulate membrane traffic from the TGN.

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