Association of a phosphatidylinositol-specific 3-kinase with a human *trans*-Golgi network resident protein

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The eukaryotic *trans*-Golgi network (TGN) is a key site for the formation of transport vesicles destined for different intracellular compartments [1]. A key marker for the mammalian TGN is TGN38/46 [2]. This integral membrane glycoprotein cycles between the TGN and the cell surface and is implicated in recruitment of cytosolic factors and regulation of at least one type of vesicle formation at the mammalian TGN [2,3]. In this study, we have identified a phosphatidylinositol (PtdIns)-specific 3-kinase activity associated with the human orthologue (TGN46), which is sensitive to lipid kinase inhibitors. Treatment of HeLa cells with low levels of these inhibitors reveals subtle morphological changes in TGN46-positive compartments. Our findings suggest a role for PtdIns 3-kinases and presumably for the product, PtdIns 3-phosphate (PtdIns3P), in the formation of secretory transport vesicles by mechanisms conserved in yeast and mammals.

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Results and discussion

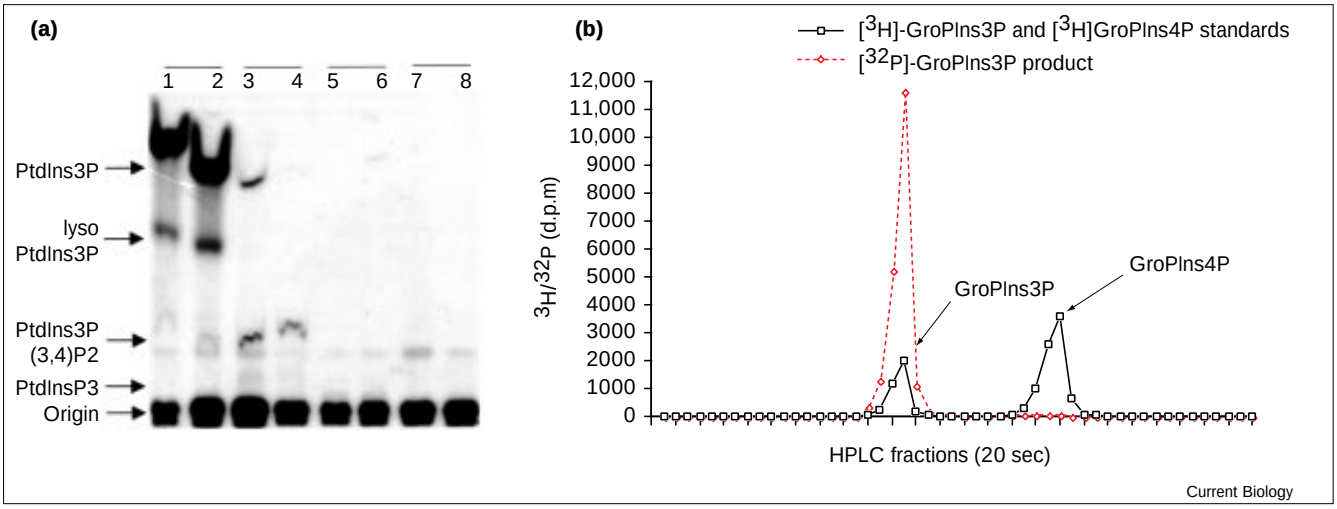
Increasing evidence implicates lipid kinases in membrane traffic events [4,5]. The TGN46 glycoprotein is associated with a specific set of cytoplasmic polypeptides [3]. TGN46 imunoprecipitates were assayed for kinase activity on different lipid substrates (Figure 1a). TGN46 complexes were found to contain a unique lipid kinase activity that phosphorylates PtdIns (Figure 1a, lanes 1,2). In contrast, phosphorylation of PtdIns 4-phosphate (PtdIns4P) was barely detectable (Figure 1a, lanes 3,4). Phosphorylation of phosphatidylinositol bisphosphate ($PtdInsP_2$) was not detected (Figure 1a, lanes 5,6). No significant lipid kinase activity specific for the PtdIns substrate was detected in cell extracts precipitated using protein A–Seppharose beads only as controls (Figure 1a, lanes 7,8). Affinity-purified sheep anti-TGN46 polyclonal antibodies also immunoprecipitate a similar lipid kinase activity from cell lysates (data not shown). Importantly, a similar, TGN46-associated PtdIns-specific kinase activity was detected in HeLa cells (data not shown). These data further support the specific association of the lipid kinase activity with the TGN46 protein complex.

The ^{32}P-labelled product was deacylated and compared with [^{3}H]glycerophosphoinositol 3-phosphate (GroPIns3P) and [^{3}H]glycerophosphoinositol 4-phosphate (GroPIns4P) standards (Figure 1b). The [^{32}P]GroPInsP had an identical retention time to that of [^{3}H]GroPIns3P. Thus the ^{32}P-labelled product generated by the TGN46-associated PtdIns kinase corresponds to PtdIns3P and hence the TGN46-associated enzyme is a PtdIns 3-kinase.

Different lipid kinases appear to have different cofactor requirements and sensitivity to inhibitors (reviewed in [4–6]). TGN46 immunoprecipitates were assayed for PtdIns 3-kinase activity in the presence of different amounts of PtdIns substrate, ATP, divalent cations and potential inhibitors and activators. The TGN46-associated lipid kinase has a K_m of 205 μM for PtdIns; the K_m for ATP is approximately 70 μM. The kinase has an absolute requirement for Mg^{2+}, which cannot be substituted for by either Ca^{2+} or Mn^{2+}. The TGN46-associated PtdIns 3-kinase was strongly inhibited by relatively low levels of both wortmannin and LY294002; the IC_{50} values are approximately 2.5 nM and 2.5 μM, respectively. Potential lipid kinase regulators such as the PtdIns transfer protein (PITP) [7] and TGN38-associated small soluble GTP-binding proteins [3] may regulate TGN46-associated PtdIns 3-kinase activity. However, neither PITP nor the non-hydrolysable GTP analogue GTPγS significantly affected enzyme activity (data not shown). Pretreatment of cells with compounds that affect TGN46 localisation, such as brefeldin A or chloroquine, had little or no effect on the levels of lipid kinase activity associated with the TGN46 complex (data not shown). Transient overexpression of TGN46 in HeLa cells [8] does not increase levels of PtdIns 3-kinase activity associated with the complex (data not shown). In fact, increased levels of TGN46 reduced the levels of kinase activity detected in immunoprecipitates by 20–40%.

Rat TGN38 is associated with a cytoplasmic complex that includes a 62 kDa phosphoprotein [3]. TGN46 complexes from human U937 cells were immunoprecipitated from

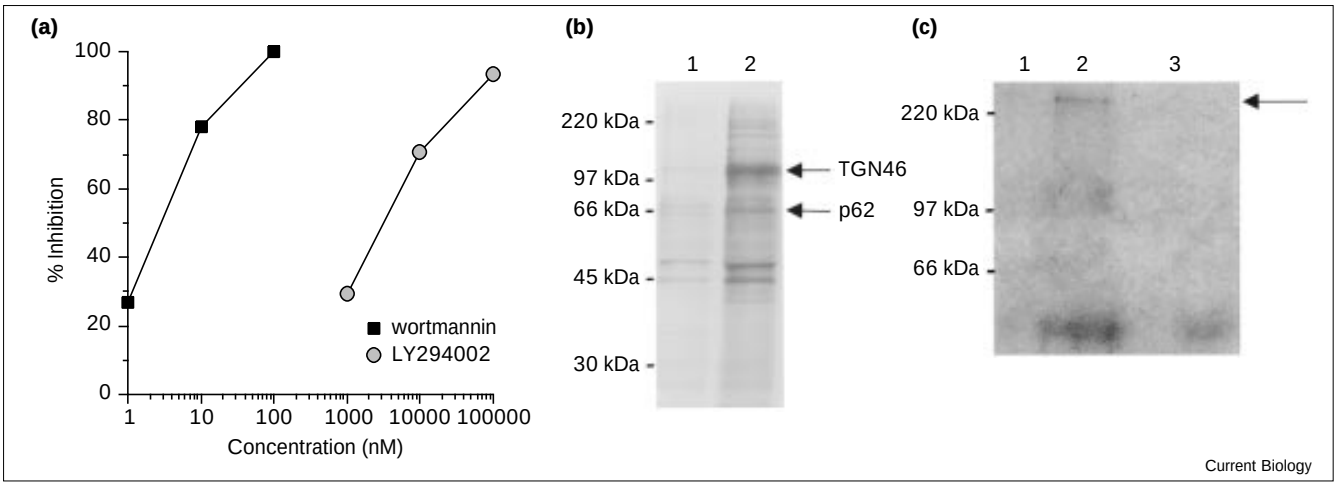
Figure 1



Identification of a PtdIns 3-kinase activity associated with TGN46. (a) Different lipid substrates were incubated with TGN46 immunoprecipitates (lanes 1–6) from U937 cells or cell extracts and protein A–Sepharose beads only (lanes 7,8), followed by thin-layer chromatography analysis. Lipid substrates tested were PtdIns (lanes 1,2,7,8), PtdIns4P (lanes 3,4) or $PtdInsP_2$ (lanes 5,6). Arrows mark the positions corresponding to the different lipid standards. (b) HPLC analysis of phosphorylated PtdIns product. HPLC profiles of ^{32}P-phosphorylated PtdIns product generated by the TGN46-associated kinase and ^{3}H-labelled standards for GroPIns3P and GroPIns4P. Samples were chromatographed on a Partisphere 5 SAX column using a linear gradient from 0–150 mM $NH_4H_2PO_4$ pH 3.8 (1 ml min^{-1}). Fractions were collected at 20 sec intervals (fraction no., *x*-axis) and counted in a scintillation counter (d.p.m., *y*-axis).

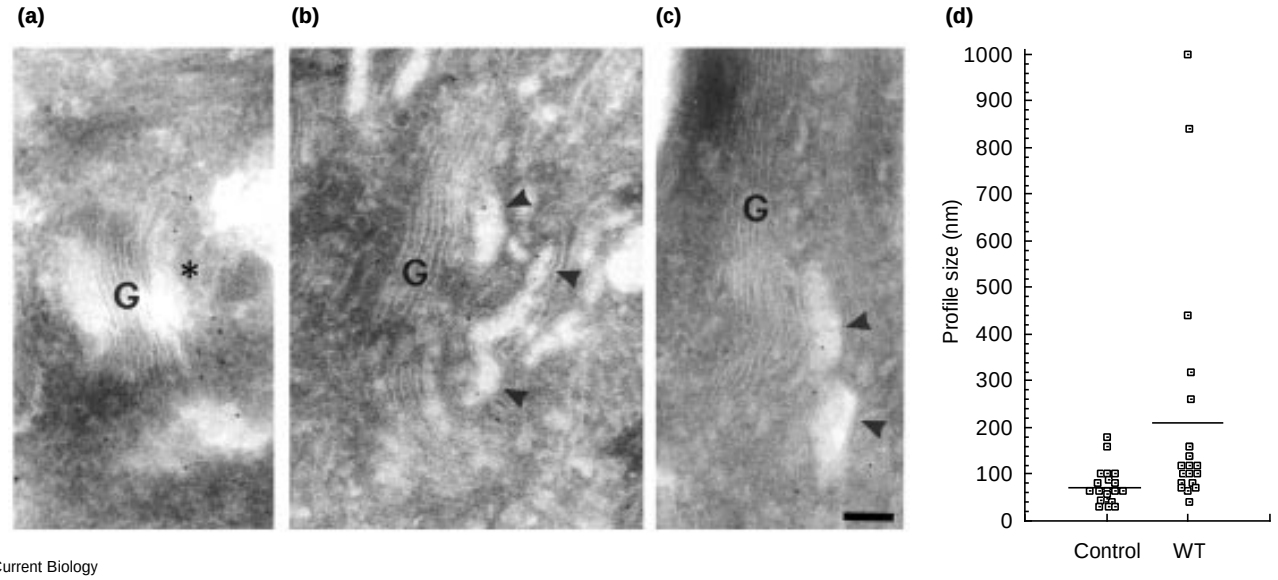
^{35}S-labelled detergent lysates and analysed by SDS–PAGE and autoradiography (Figure 2b). Comparison of control immunoprecipitates using rabbit pre-immune serum (Figure 2b, lane 1) and anti-TGN46 polyclonal antibodies reveals a discrete set of polypeptides associated with the TGN46 complex; this includes the TGN46 glycoprotein, a 62 kDa polypeptide (arrow in Figure 2b, lane 2) and high-molecular-weight polypeptides at around

Figure 2



Biochemical properties of TGN46-associated polypeptides. (a) Wortmannin and LY294002 inhibit TGN46-associated PtdIns 3-kinase activity. Immunoprecipitates were assayed for the production of PtdIns3P in the presence of different concentrations of wortmannin or LY294002. The relative percentage of enzyme activity detected was plotted against a log scale of concentrations of wortmannin or LY294002. Each point on the graph is a mean of two duplicate experiments. (b) ^{35}S-labelled TGN46 complexes were immunoprecipitated from U937 cells, fractionated on 10% SDS–PAGE gels, dried, and analysed by autoradiography. (c) Immunoprecipitates bound to control protein G–Sepharose beads (lane 1) or affinity-purified sheep anti-TGN46 antibodies bound to protein G–Sepharose (lanes 2,3) were treated with [^{3}H]wortmannin in the absence (lanes 1,2) or presence (lane 3) of 100-fold excess of cold wortmannin, fractionated on 8% SDS–PAGE gels and analysed by autoradiography.

Figure 3



Effects of wortmannin and LY294002 on the TGN46-labelled compartment. Thin cryosections of control HeLa cells (a) or cells treated with 50 μM LY294002 (b) or 100 nM wortmannin (c) were labelled with rabbit polyclonal antibodies to TGN46 followed by protein A–6 nm gold conjugates. Arrowheads in (b) and (c) point to swollen electron-lucent structures labelled for TGN46 on the *trans* side of the Golgi stack. The *trans*-Golgi network , asterisk in (a), and the Golgi stack, G in (a–c), are labelled. Scale base in (c) = 1 μm. (d) A representative number of TGN46-labelled profiles from untreated (control) and wortmannin (WT)-treated HeLa cells were analysed and the diameter of each profile is represented. The mean profile size in each experiment is indicated by the horizontal bar for each set of values (see text).

200–250 kDa (Figure 2b, lane 2). Similar results were obtained from immunoprecipitations using affinity-purified sheep anti-TGN46 polyclonal antibodies (data not shown). To identify wortmannin-sensitive catalytic subunits in the TGN46 complex, immunoprecipitates from U937 cells were treated with ^{3}[H]wortmannin (Figure 2c) in the absence (lane 2) or presence (lane 3) of excess cold wortmannin. No labelled polypeptides are visible in the control immunoprecipitation (lane 1). However, a tritium-labelled, high-molecular-weight polypeptide of approximately 250 kDa is visible upon treatment of the immunoprecipitated TGN46 complex with [^{3}H]wortmannin (Figure 2c, lane 2).

HeLa cells treated with either wortmannin or LY294002 were analysed using light fluorescence microscopy; we did not detect any significant overall effects on TGN46 localization, in agreement with previous studies [9,10]. However, treatment with wortmannin or LY294002 did cause accumulation of the transferrin receptor in endosomes, again in agreement with previous studies [10] (data not shown). To look for effects at the ultrastructural level, HeLa cells treated with 100 nM wortmannin were processed for immuno-gold localisation of TGN46 on ultrathin cryosections. In untreated controls, TGN46 is localised over a compact accumulation of tubulovesicular profiles close to the Golgi stack (Figure 3a); this compartment appears to be a mixture of tubules, vesicles and cisternal membranes [11,12]. Upon treatment with wortmannin (Figure 3c) or LY294002 (Figure 3b), TGN46-positive structures tended to appear more dilated and electron lucent (Figure 3b,c, arrowheads).

TGN46-labelled profiles were further analysed by measuring the diameter of both electron-dense and electron-lucent structures (Figure 3d). In untreated controls, the TGN46-labelled profiles generally ranged in size between 50 and 150 nm in diameter (mean 70 nm; coefficient of error = 12%, n = 20). To further examine wortmannin effects on Golgi structure under these conditions we also processed cells for conventional epoxy resin thin-section analysis. Although electron-lucent profiles close to the Golgi stack were detected in wortmannin-treated cells, the effect was less marked than in cryosections (results not shown).

The effects of wortmannin treatment were not observed in every Golgi apparatus profile, but quantitation showed a marked increase in the fraction of the TGN46 labelling over electron-lucent structure profiles from 27% (coefficient of error = 29%, n = 20) in controls to 43% (coefficient of error = 26%, n = 21) upon

wortmannin treatment. These swollen structures may be derived from endosomal membranes or correspond to an expansion of the TGN46-positive compartment in the TGN. As these inhibitors affect several lipid kinases [4–6], we cannot be certain that these effects are due to the TGN46-associated enzyme. An alternative is that PtdIns 3-kinase inhibition dramatically increases compartment volume by inhibition or activation of transporters such as proton pumps.

Increasing evidence suggests that PtdIns-specific 3-kinases regulate membrane traffic events in the endocytic pathway and from the TGN to distal compartments in eukaryotes [4,6]. An important example is the *Saccharomyces cerevisiae VPS34* gene product, which regulates transport of carboxypeptidase Y from the Golgi apparatus to the yeast vacuole [4]; the TGN46-associated PtdIns 3-kinase may be involved in a similar step in membrane traffic from the TGN to a distal compartment. A variety of PtdIns-specific 3-kinases have been cloned from eukaryotes and encode polypeptides ranging from 100 kDa–190 kDa (reviewed in [6]). Vps34 is a 100 kDa polypeptide with PtdIns 3-kinase activity that is inhibited only by relatively high micromolar concentrations of wortmannin [4]. However, in contrast to yeast Vps34, most of the other mammalian PtdIns 3-kinases are sensitive to low nanomolar concentrations of wortmannin. We have identified a [^{3}H]wortmannin-labelled polypeptide of approximately 250 kDa in the TGN46 complex; this is likely to account for the PtdIns 3-kinase activity associated with such complexes and is a candidate for the wortmannin and LY294002 target *in vivo*. Once sequences of this polypeptide are obtained, it will be possible to determine whether it is a new or a previously identified member of the PtdIns 3-kinase family. Preliminary work by Howell and co-workers [13] suggests that a lipid kinase activity is also associated with the rat orthologue, TGN38. Cloning of the TGN46-associated PtdIns 3-kinase enzyme described in this study, combined with biochemical and cell biological investigations, will provide new insights into the molecular principles governing such membrane dynamics.

Materials and methods

Immunoprecipitations, lipid kinase assays and electron microscopy

TGN46 complexes were immunoprecipitated from U937 or HeLa cell lysates as previously described [3] using rabbit anti-TGN46 [11] or affinity-purified sheep anti-TGN46 (M.T. and S.P., unpublished observations) polyclonal antibodies. Lipid kinase assays were carried out as previously described [14] and all experiments were carried out in duplicate. Control immunoprecipitations using an equivalent amount of preimmune serum also showed less than 0.5% kinase activity (relative to TGN46 immunoprecipitates) using PtdIns as a substrate. Deacylation of lipids and subsequent analysis of the products were carried out using base catalysis and HPLC [15]. Cells were treated for 2 h with either of the lipid kinase inhibitors and processed for immuno-electron microscopy as previously described [11,12].

Supplementary material

Further details of methodology and a table showing the biochemical properties of the TGN46-associated PtdIns 3-kinase are published with this paper on the internet.

Acknowledgements

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