

## Localization of a Class II Phosphatidylinositol 3-Kinase, PI3KC2 $\alpha$ , to Clathrin-Coated Vesicles

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**We have analysed phosphatidylinositol 3-kinase activity associated with subcellular fractions prepared from rat brains. Phosphatidylinositol 3-kinase activity is not markedly enriched with synaptic vesicle purification; whilst the activity associated with the most pure fractions is inhibited at low concentrations of wortmannin ( $IC_{50} \sim 4\text{--}5$  nM). In contrast, clathrin-coated vesicle (CCV) fractions showed increased enzyme activity compared to light membrane fractions from which they are purified. In addition to a wortmannin-sensitive activity, we also detected an activity that could only be inhibited at higher concentrations of wortmannin ( $IC_{50} \sim 400$  nM), characteristic of certain class II enzymes (including phosphatidylinositol 3-kinase C2 $\alpha$ ) to be highly enriched in CCV fractions. Immunoblotting with an antibody raised against phosphatidylinositol 3-kinase C2 $\alpha$ , confirmed that this enzyme is highly enriched in CCVs and displays an enrichment profile during the purification that mirrors enrichment of the low nanomolar wortmannin-insensitive activity. If the CCV purification protocol is adapted to favour nerve terminally derived vesicles, we find reduced levels of the C2 $\alpha$  enzyme in the CCV fractions, suggesting that the enzyme may principally reside on vesicles associated with the cell body.** © 1999 Academic Press

Phosphorylation of inositol lipids at the D-3 position by phosphatidylinositol 3-kinase (PI3-kinase) plays an important role in a wide range of cellular processes. These include regulation of apoptosis, differentiation, membrane ruffling and cytoskeletal rearrangement. In addition to classical signal transduction pathways, a requirement for PI 3-kinase activity in regulating constitutive membrane trafficking events has been shown (1).

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There are at least nine members of the PI3K family in mammalian cells, that have been classified into three groups according to their substrate preferences (2). Class I PI3-kinases were the first to be characterised, they include the heterodimeric p85/p110 $\alpha$ , p85/p110 $\beta$  and p101/p110 $\gamma$ . They can utilise phosphatidylinositol (PI), PI(4)P and PI(4,5)P<sub>2</sub> as substrates *in vitro*, although there is evidence that PI(4,5)P<sub>2</sub> represents the major physiological substrate (3, 4). All members of this class are sensitive to inhibition by the fungal metabolite wortmannin, ( $IC_{50} < 10$  nM). These PI3-kinases bind tyrosine phosphorylated receptors (p85/p110 $\alpha$ , p85/p110 $\beta$ ) or  $\beta\gamma$  sub-units of heterotrimeric G proteins (p110 $\gamma$ ) and are involved in signal transduction pathways.

Class II PI3-kinases can phosphorylate PI and PI(4)P *in vitro* and have variable responses to wortmannin, for example HsC2-PI3K has an  $IC_{50} \sim 10$  nM, whereas PI3KC2 $\alpha$  has an  $IC_{50}$  of 420 nM (5, 6). They are all characterised by the presence of a C-terminal C2 domain of the type that binds phospholipids in a Ca<sup>2+</sup>-independent manner (7). Roles for class II kinases in integrin signalling in platelets and for pertussis toxin-sensitive signalling in response to monocyte chemoattractant peptide-1 in THP-1 cells have been reported (5, 8).

Class III PI3-kinases are exemplified by the yeast VPS34 gene product. Vps34p is required for TGN-vacuolar traffic; it is specific for PI and is wortmannin-insensitive (9)). A human homologue has been identified that is also restricted to PI as a substrate, but is sensitive to wortmannin ( $IC_{50} = 5\text{--}10$  nM; (10)). PI(3)P the product of Vps34 activity binds to a double zinc finger domain in effector proteins, referred to as a FYVE domain which can localise proteins with this domain to membranes (11, 12, 13). The major role of this class of enzymes is likely to be the regulation of endocytic membrane traffic.

Little is known about the subcellular localisation of the various enzymes. The typically low levels of endogenous protein are not conducive to immunohistochemistry, whilst overexpression leads to uncertainty. In

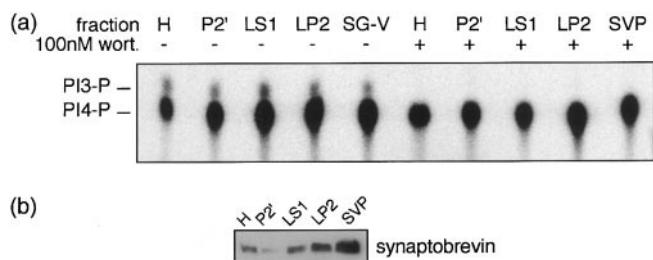
certain favourable cases subcellular fractionation protocols allow the preparation of virtually pure defined fractions which can be used for analysis of protein composition. We have taken advantage of well established protocols for purification of vesicles from brain homogenate (14, 15) to examine associated PI 3-kinase activity. A similar approach has previously been used to localise other key enzymatic activities, for example PI4-kinase activity has been found to be enriched on synaptic vesicles (16). We find no specific enrichment of PI 3-kinase activity with synaptic vesicles but find specific enrichment of PI3KC2 $\alpha$  on whole brain derived clathrin coated vesicles (WB-CCVs).

## MATERIALS AND METHODS

### Vesicle Preparation

Synaptic vesicles were prepared according to the method of Huttner *et al.*, and their terminology has been adopted (14). Briefly, synaptosomes (P2' fraction) were isolated from brain homogenate. Following lysis of the synaptosomes by osmotic shock, a 20 minute, 25,000g spin produced a lysate supernatant (LS1), which was then pelleted (165,000g, 2 hours) to produce the LP2 fraction enriched in synaptic vesicles. The pellet was resuspended and loaded onto a linear continuous sucrose gradient (50-800 mM). Following centrifugation (5 hours, 65,000g) the SG-V fraction was harvested as a turbid band in the 0.2-0.4 M sucrose region. The SG-V fraction was diluted in an equal volume of ice cold distilled water and then spun at 185,000g<sub>av</sub> for 2 hours, 4°C (TFT 55.38 rotor; Kontron Instruments). The final synaptic vesicle pellet (SVP) was resuspended in buffer S (0.32 M sucrose, 10 mM EGTA, 4 mM HEPES/NaOH, pH 7.3), snap frozen and stored at -70°C.

Clathrin-coated vesicles (CCVs) were prepared from whole rat brain homogenate or isolated rat synaptosomes as described (15). Briefly, for whole brain isolated CCVs, a light membrane fraction (LM) was produced from brain homogenate by differential centrifugation steps. The light membranes were sub-fractionated by layering over a 12.5% Ficoll cushion and collecting the supernatant (PFS) after a 40,000g spin. Following further differential centrifugation steps to remove substantial smooth membrane contamination an enriched CCV supernatant fraction (EF) was produced. The EF was loaded onto the final D<sub>2</sub>O-8% sucrose step gradient to yield a highly purified CCV pellet following centrifugation. For isolation of CCVs from nerve terminals a crude synaptosome preparation is first prepared, washed to remove contaminating smooth membrane and ruptured using osmotic shock. The lysate was adjusted to iso-osmolarity and then taken through the same steps as for the brain homogenate in the WBCCV procedure (15).



**FIG. 1.** PI 3-kinase activity throughout synaptic vesicle purification. (a) 5  $\mu$ g of material from each step of the synaptic vesicle purification procedure was assayed for PI 3-kinase activity  $\pm$  100 nM wortmannin. Assay procedures and nomenclature of fractions are described in Materials and Methods. H, homogenate is the crudest material whilst SG-V represents the purest fraction. No enrichment of PI 3-kinase activity is observed that corresponds to enrichment for the synaptic vesicle marker synaptobrevin that is shown by Western blotting of fractions in (b). PI 3-kinase activity in each fraction is completely inhibited by 100 nM wortmannin.

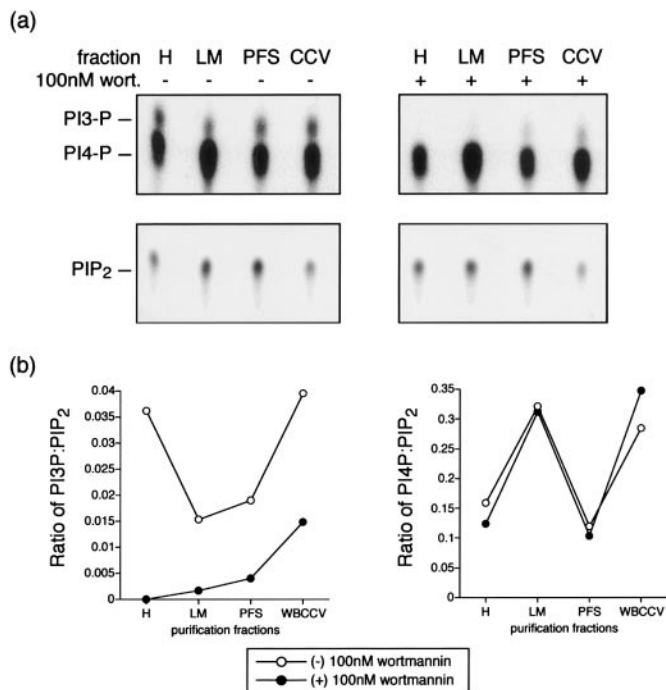
The final whole brain CCV (WBCCV) and nerve terminal CCV (NTCCV) pellets were resuspended in buffer A (0.1 M MES/NaOH, pH 6.5, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>), snap frozen and stored at -70°C.

### PI3-Kinase Assay

The PI3-kinase assay of (17) was adapted for use with vesicle fractions. 1  $\mu$ l of 20 mg/ml phosphatidylinositol in DMSO was added to vesicle fractions (5  $\mu$ g) in 50  $\mu$ l reaction buffer. If wortmannin was included in the incubation it was also added at this point to the indicated final concentrations. Following incubation for 10 minutes at 25°C; 1  $\mu$ l of ATP stock solution (3.8 nM <sup>32</sup>P-ATP, 9.4  $\mu$ M ATP) and 1  $\mu$ l of 1 M MgCl<sub>2</sub> (were added and incubated for 30 minutes at 25°C. The reaction was stopped by addition of 100  $\mu$ l of freshly prepared chloroform:methanol:10 M HCl (100:200:2). The organic phase was collected after separation from the aqueous phase by centrifugation. A borate based thin layer chromatography method was used for separation of phosphorylated lipids (18). 25  $\mu$ l of the organic phase was spotted onto a silica plate using a gel loading pipette tip. Following chromatography the image of the dried plates was developed in a phosphorimager cassette for 24 hours.

### Immunoblotting

Western blots were probed with mouse anti-synaptophysin (Sigma), rabbit anti-synaptobrevin, rabbit anti-PI3 kinase C2 $\alpha$  (8), mouse anti  $\alpha$ -adaptin (Sigma), rabbit anti- $\gamma$ -adaptin (19). Following incubation with horseradish peroxidase conjugated secondary antibodies (1:1000 dilution) the proteins were visualised using a chemiluminescence detection system (Pierce Super-signal; Pierce and Warriner).



**FIG. 2.** PI 3-kinase and PI 4-kinase activity throughout the purification of CCVs from rat brain. 5  $\mu$ g of material from each step of the synaptic vesicle purification procedure was assayed for phosphatidylinositol kinase activity  $\pm$  100 nM wortmannin. Assay procedures and nomenclature of fractions are described in Materials and Methods. (a) reveals that in the pure CCV fraction a significant amount of PI 3-kinase activity remains in the presence of 100 nM wortmannin. This is quantitated in (b) where the PI3P and PI4P generated by each fraction have been ratioed against the PIP<sub>2</sub> that is generated.

## RESULTS AND DISCUSSION

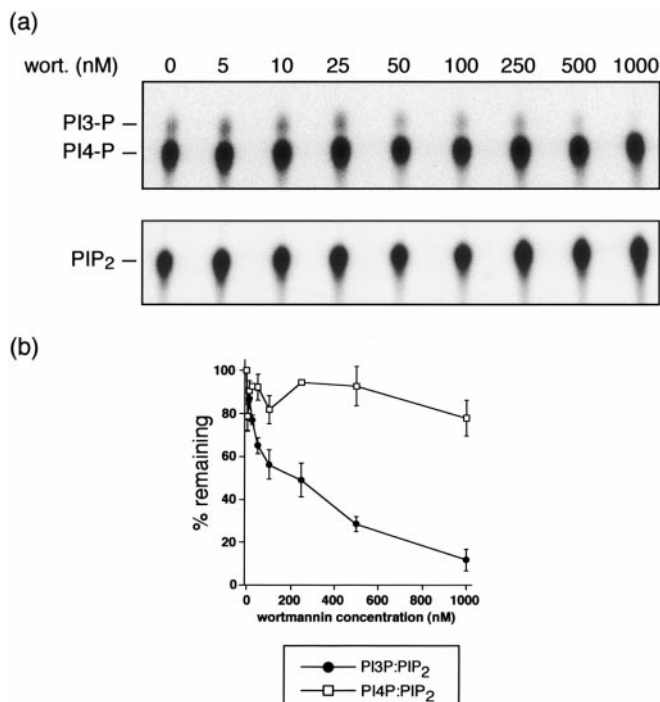
### Distribution of Lipid Kinase Activities

Lipid kinase activities of enriched synaptic vesicle and CCV fractions were measured by incubating the fractions with phosphatidylinositol micelles and <sup>32</sup>P-ATP to allow the kinases to generate radiolabelled PI species. The lipids were extracted and separated using thin layer chromatography and the radiolabelled products were quantitated using a phosphorimager and associated software. The method allows the separation of PI(3)P, PI(4)P and PIP<sub>2</sub>. In all cases PI(4)P was at least 10-fold more abundant than PI(3)P and PIP<sub>2</sub> was typically 5-10-fold more abundant than PI(4)P. We used equal protein concentrations to perform the kinase assays. However, we have also used PIP<sub>2</sub> levels as an internal standard for normalisation purposes to represent relative enrichment of PI 3-kinase activity in an alternative manner. One reason for this is that the proportion of membrane proteins in each fraction will vary, particularly in more highly purified CCV fractions. Coat proteins represent approximately 70% of total protein in a CCV fraction. The majority of cellular

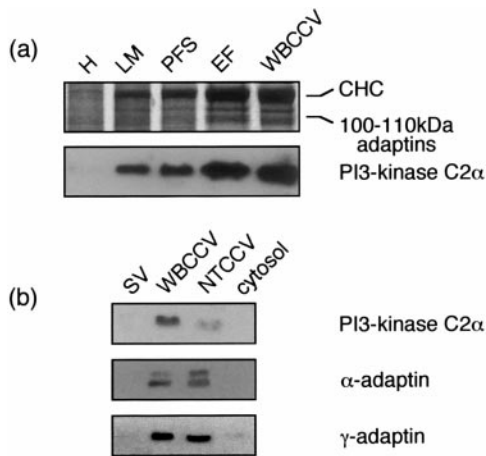
PIP<sub>2</sub> is PI(4,5)P<sub>2</sub> therefore the activity of PI3-kinase should not greatly influence the PIP<sub>2</sub> pool.

PI3-kinase activity is associated with a synaptic vesicle fraction (SVP; Figure 1(a)); however, no significant pattern of enrichment is observed through the preparation from homogenate. It is not possible to exclude the possibility that the activity that we observe is associated with contaminating membranes. This activity is completely inhibited by 100nM wortmannin (IC<sub>50</sub> ~ 5 nM, data not shown). A different picture emerges through the CCV preparation. Although a 60% reduction in PI3-kinase activity is observed in the light membrane fraction (LM) relative to homogenate; a substantial enrichment of PI3-kinase activity is subsequently observed through the remaining purification (Figure 2). Only 60% of the PI 3-kinase activity associated with the pure CCV fraction is inhibited by 100 nM wortmannin. The remaining wortmannin insensitive PI 3-kinase activity is highly enriched from the starting material where it is not detectable (Figure 2); it is enriched approximately 9 fold over the light membrane fraction (normalised for PIP<sub>2</sub> production).

The wortmannin dose response of whole brain clathrin coated vesicle PI 3-kinase activity is presented in detail in Figure 3. It is clearly biphasic; the IC<sub>50</sub> of the less sensitive component is approximately 400 nM. This is consistent with the properties of certain class II enzymes. The IC<sub>50</sub> value reported for human PI



**FIG. 3.** Wortmannin sensitivity of WB-CCV lipid kinases. 5  $\mu$ g of CCVs was assayed for lipid kinase activity  $\pm$  the stated concentrations of wortmannin. PIP<sub>2</sub> levels were used as an internal standard to correct for any variation in loading of each lane.



**FIG. 4.** Distribution of PI3KC2 $\alpha$  in rat brain subcellular fractions: (a) top panel shows coomassie stained gel indicating enrichment of clathrin and adaptins during CCV preparation; bottom panel shows a Western blot indicating parallel enrichment of PI3KC2 $\alpha$  throughout the preparation. Equal amounts of protein were loaded in each lane. Nomenclature of fractions is described in Materials and Methods. (b) Western blots using antibodies against indicated proteins for 7  $\mu$ g each of synaptic vesicles (SV), whole brain CCVs (WBCCV), nerve terminal biased CCVs (NTCCV), and cytosol. The two CCV fractions exhibit differing relative amounts of adaptin proteins, in particular the difference in isoforms of  $\alpha$ -adaptin is striking. Nerve terminal CCVs contain less PI3KC2 $\alpha$ .

3-kinase C2 $\alpha$  (6), which falls in the same range, prompted us to test directly for the presence of this enzyme by Western blotting of the fractions. Figure 4 shows that this isoform is indeed enriched throughout the CCV preparation in a pattern that mirrors the enrichment observed for a wortmannin-insensitive PI 3-kinase activity (Figure 2). No PI3KC2 $\alpha$  could be detected in synaptic vesicle fractions by Western blotting.

In distinction to other PI 3-kinase isoforms which are in dynamic equilibrium between particulate and cytosolic fractions, PI3KC2 $\alpha$  is proposed to be permanently bound to membrane fractions. We have shown above that PI3KC2 $\alpha$  is found on CCVs purified from whole brain but not synaptic vesicles. If it is present on nerve terminal-derived CCVs this would have implications for models of synaptic vesicle recycling. It would argue against the simplest model of recycling in which clathrin coated vesicles simply uncoat to produce clathrin coated vesicles (20, 21). We have addressed this issue by modifying our CCV preparation to bias it towards the purification of nerve terminal derived vesicles, by first preparing a crude synaptosomal fraction from rat brain. In our hands these NT-CCVs are 2-3 fold more enriched over WB-CCVs with the synaptic vesicle marker synaptophysin (21). Figure 4B shows the relative PI3KC2 $\alpha$  content between NT-CCVs, WB-CCVs and synaptic vesicles. For comparison the relative contents of adaptor proteins  $\alpha$  and  $\gamma$ -adaptin are shown. The difference between the two CCV fractions is highlighted by the relative amounts of different

$\alpha$ -adaptin isoforms (Figure 4B). PI3KC2 $\alpha$  is less enriched in the nerve terminal-biased fraction, thereby indicating an association of the enzyme with CCVs in the cell body rather than the nerve terminal. We have not been able to detect any PI3KC2 $\alpha$  in the cytosol, even using long exposure times.

Our approach does not allow us to distinguish with which class of cell body CCVs PI3KC2 $\alpha$  is associated with. The relevant vesicles are most likely to be of plasma membrane or trans-Golgi network origin (AP2 or AP1 adaptor complexes respectively). It is possible that enzyme activity may be required for intra-cellular trafficking. CCV-dependent trafficking of cathepsin D from the TGN to the endosomal network is inhibited by wortmannin at variable doses depending on the cell line (10-3000 nM (22, 23, 24)). Fluid phase endocytosis can also be inhibited by wortmannin (25, 26), although there is now evidence that this may be due to inhibition of non-clathrin dependent uptake pathways (reviewed in (27)). The location of the enzyme may reflect participation in sorting events or vesicle formation, although the class III enzyme hVPS34 is also a strong candidate for regulating vesicular traffic. We currently favour the view that association of PI3KC2 $\alpha$  activity with a vesicular intermediate may reflect the requirement for elements of an associated signal transduction apparatus to traverse this route, in order to exert its full range of physiological effects. For example EGF receptor signalling has been shown to depend upon internalisation via clathrin coated vesicles (28).

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