Identification and characterization of differentially active pools of type II α phosphatidylinositol 4-kinase activity in unstimulated A431 cells

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The seven known polyphosphoinositides have been implicated in a wide range of regulated and constitutive cell functions, including cell-surface signalling, vesicle trafficking and cytoskeletal reorganization. In order to understand the spatial and temporal control of these diverse cell functions it is necessary to characterize the subcellular distribution of a wide variety of polyphosphoinositide synthesis and signalling events. The predominant phosphatidylinositol kinase activity in many mammalian cell types involves the synthesis of the signalling precursor, phosphatidylinositol 4-phosphate, in a reaction catalysed by the recently cloned PI4KII α (type II α phosphatidylinositol 4-kinase). However the regulation of this enzyme and the cellular distribution of its product in different organelles are very poorly understood. This report identifies the existence, in unstimulated cells, of two major subcellular membrane fractions, which contain PI4KII α possessing different levels of intrinsic activity. Separation of these

membranes from each other and from contaminating activities was achieved by density gradient ultracentrifugation at pH 11 in a specific detergent mixture in which both membrane fractions, but not other membranes, were insoluble. Kinetic comparison of the purified membrane fractions revealed a 4-fold difference in K_m for phosphatidylinositol and a 3.5-fold difference in V_{max} , thereby indicating a different mechanism of regulation to that described previously for agonist-stimulated cells. These marked differences in basal activity and the occurrence of this isozyme in multiple organelles emphasize the need to investigate cell signalling via PI4KII α at the level of individual organelles rather than whole-cell lysates.

Key words: detergent-insoluble membrane, low-buoyant-density membrane, membrane microdomain, phosphatidylinositol kinase, phosphoinositide signalling, subcellular fractionation.

INTRODUCTION

The synthesis of PI4P (phosphatidylinositol 4-phosphate) by the phosphorylation of PI (phosphatidylinositol) on the 4' position of the inositol residue is an essential step in the biochemical pathway that produces PtdIns(4,5) P_2 for receptor-dependent phospholipase C and phosphoinositide 3-kinase signalling [1–3]. In addition, PI4P acts at cellular membranes as a binding partner, for example for PI4P-specific pleckstrin homology and ENTH (epsin N-terminal homology) domain-containing proteins [4–6]. Two families of enzymes have been cloned that specifically catalyse the conversion of PI to PI4P: the adenosine-sensitive type II PI 4-kinases (PI4KII) and the wortmannin-sensitive type III PI 4-kinases (PI4KIII) [7].

Type II activity has been found in numerous subcellular organelles and vesicles, although recent results demonstrate that the majority of the PI4KII α protein is localized to the Golgi compartment, while PI4KII β translocates between the cytosol and plasma membrane [8]. PI4KIII α (p230) has been found at the plasma membrane and ER (endoplasmic reticulum), while PI4KIII β (p92) translocates between the cytosol and Golgi compartment [7]. Studies on phosphoinositide signalling have commonly relied on assays of net PI4P levels and PI4K activity, and the activity and specific functions of different PI4K isozymes in different subcellular membranes, particularly in mammalian cells, are not at all clear.

In A431 cells measurable PI4P production is mainly accounted for by a pool of active PI4KII α contained within low-buoyant-density membranes, which have a protein composition similar

to the ER [9]. These membranes are particularly rich in VCP (valosin-containing protein), an AAA + ATPase (ATPase associated with a variety of cellular activities, and with a hexameric ring structure), but their cellular function is unknown. The bulk of the PI4KII α protein in these cells has been localized mainly to the Golgi compartment using microscopy and subcellular fractionation techniques and appears to be in a less active form [8,9]. No enzymic comparison of co-existing pools of PI4KII α has previously been reported.

A major reason for the absence of any comparative analysis of the different subcellular pools of PI4KII α is their incomplete resolution using ultracentrifugation [9]. Furthermore, although PI4KII activity was previously localized to a buoyant, non-caveolar membrane fraction of A431 cells [10], it was unclear whether this fraction contained one or both pools of PI4KII α activity. A second reason is that other PI4K isozymes had not been separated from either pool of PI4KII α , and therefore contributed to the measured activity. Consequently, removal of the other PI4K isozymes was required before any meaningful comparison of type II α activities was possible.

This report describes a method for the separation of the two pools of PI4KII α -containing membranes from each other and from other PI4K isozymes, and a quantitative characterization of enzymic differences between the PI4KII α in these two membrane pools. The recognition of different membrane pools containing PI4KII α of widely different basal activity combined with the development of a method to resolve the major pools will allow far better informed and more meaningful analyses of the regulation of this enzyme to be performed in future.

Abbreviations used: β -OG, β -octylglucoside; DOC, deoxycholate; ER, endoplasmic reticulum; PI, phosphatidylinositol; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PIPK, phosphatidylinositol 4-phosphate 5-kinase; VCP, valosin-containing protein; GST, glutathione S-transferase; EGFR, epidermal growth factor receptor; PITP, phosphatidylinositol-transfer protein.

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EXPERIMENTAL

Materials

Anti-PI4KII α antiserum [9] for use in immunoblotting was purified against recombinant GST (glutathione S-transferase)-PI4KII α using a Western blotting-based protocol essentially as described in [11], but employing Immobilon-P (Millipore) instead of nitrocellulose. For use in immunoprecipitation the same antiserum was purified on Protein A-Sepharose CL4B (Amersham Biosciences). Anti-PI4KII β antiserum was raised in New Zealand White rabbits using full-length recombinant GST-PI4KII β (S. Minogue and J. J. Hsuan, unpublished work). Anti-PI4KII β antiserum was purified using the Western blotting procedure prior to use in immunoblotting. Anti-calnexin was purchased from Stress-Gen, anti-EGFR (epidermal growth factor receptor) peptide antiserum (anti-2E) has been described previously [12]. Anti-PI4KIII α was kindly provided by Dr J. Backer (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) and anti-PI4KIII β was purchased from Upstate Biotechnology. Antisera to the PIPKI (PI4P 5-kinase type I; PIPKI α) and to the PITP (PI-transfer protein; PITP α) were purchased from Santa Cruz Biotechnology. Anti-VCP antiserum was from Pharmingen (BD Biosciences). Protein A-Sepharose CL-4B, ECL Western blotting system [13], inositol with PT6-271 stabilizer (17.1 Ci/mmol) and [γ -³²P]ATP (4500–6000 Ci/mmol) were from Amersham Biosciences. Protease inhibitor cocktail tablets (Complete, without EDTA) were from Roche Diagnostics.

Cell culture and metabolic radiolabelling

A431 cells were cultured, labelled for 48 h to reach equilibrium using [³H]inositol, and analysed using TLC as described previously [9].

PI4K activity assays

PI4K assays in the presence of endogenous and exogenous PI were performed as described previously [10]. Reaction products were separated by TLC and visualized on a Typhoon 9400 phosphorimager (Amersham Biosciences). Quantitative data were obtained using ImageQuant Software. Michaelis–Menten kinetic analyses were performed by non-linear regression curve fitting using Prism software (GraphPad) and compared using the unpaired Student's *t* test. Western blotting of fractions indicated that equivalent levels of PI4KII α were present in the assayed buoyant and dense fractions thus allowing for comparisons of apparent $V_{\max}$, for both samples. One of the assumptions of Michaelis–Menten kinetic analyses is that both enzyme and substrate are freely diffusible. As substrate PI is present in micellar form in the assays, it is not strictly a freely diffusible substrate, hence the resultant $V_{\max}$ and K_m values obtained represent apparent values.

Subcellular fractionation

Separation of A431 cell post-nuclear supernatants on a continuous 10–40 % (w/v) sucrose gradient was performed as described previously [9]. For agonist stimulations cells were serum-starved overnight in Dulbecco's modified Eagle's medium supplemented with 1 % fetal calf serum. Agonist stimulations were carried out at 37 °C and the reactions stopped by placing the cells on ice and rapid aspiration. Cell homogenization, generation of post-nuclear supernatants and separation of cell membranes on a discontinuous 10–40 % sucrose gradient were as previously reported [10].

Isolation of β -OG (β -octylglucoside) and DOC (deoxycholate)-insoluble membranes

The isolation of detergent-insoluble PI4P-synthesizing membranes was carried out as follows. A431 cell monolayers were washed twice with ice-cold PBS (pH 7.4) prior to the addition of 2 ml of 10 mM β -OG and 4 mM DOC (β -OG/DOC) in 100 mM Na₂CO₃, 10 mM EDTA and 10 mM EGTA, pH 11, containing Complete protease inhibitor cocktail. Cells were harvested by scraping into a test tube and sonicated for six 5 s blasts using a Vibra-Cell sonicator (Sonics) at amplitude setting 60. The resulting sonicate was mixed with 90 % (w/v) sucrose in 20 mM Tris/HCl, pH 7.4 and layered in the bottom of a 12 ml ultracentrifuge tube. A discontinuous sucrose gradient consisting of 4 ml of 35 % (w/v) sucrose followed by 4 ml of 5 % (w/v) sucrose was then layered on top. The tube was centrifuged overnight at 175 000 *g*. After centrifugation, 1 ml fractions were collected beginning at the top of the tube.

Equal volumes of either low-buoyant-density fractions 4 and 5 or of fractions 11 and 12 from the β -OG/DOC sucrose gradient were pooled and separated from the gradient buffer by centrifugation at 100 000 *g* for 45 min using a Optima MAX bench-top ultracentrifuge (Beckman-Coulter). The supernatants were decanted and the β -OG/DOC-insoluble pellets dissolved in 2 $\times$ PI4K assay buffer [0.8 % (v/v) Triton X-100, 20 mM Tris/HCl, 20 mM MgCl₂ and 0.2 mM EGTA, pH 7.4]. Following gentle mixing and re-suspension of the pellets the solutions were cleared at 100 000 *g* for 45 min before being used in kinetic studies.

Immunoprecipitation of PI4KII α

Protein A-purified anti-PI4KII α antiserum was added at a dilution of 1:100 to soluble PI4KII α prepared for use in kinetic experiments (see above) and incubated for 1 h at 4 °C. PI4KII α immunocomplexes were then precipitated using Protein A-Sepharose CL-4B.

Immunoblotting of subcellular fractions

Samples were mixed with an equal volume of 2 $\times$ sample buffer and separated by SDS/PAGE. Proteins were transferred to Immobilon-P membranes and probed with various antibodies. Bound antibody was detected using the ECL system (Amersham Biosciences).

RESULTS

Separation of subcellular organelles prepared from homogenized A431 cells using ultracentrifugation on a continuous density gradient had previously revealed a single major peak of PI4KII α activity localized to a low-buoyant density, VCP-rich fraction and an overlapping but non-corresponding peak of PI4KII α protein in higher density fractions. The extent of overlap varied between experiments, probably due to small differences in gradient formation and fraction collection (see [9] and Figures 1a and 1b). In these experiments the contributions made by PI4KII β and the two PI4KIII isozymes to the apparent activity, particularly of the higher density fraction, were unclear. Also unclear was the potential effect of endogenous PITP and PIPK activities on the measured PI4K activity.

The location within these gradients of type II β PI4K, PITP and PIPK was unknown. While Western blotting revealed that the type III β PI4K (p92) was well resolved from both the PI4K-containing fractions, the bulk of the type III α PI4K (p230) was

Figure 1 Subcellular fractionation of homogenized A431 cells on a continuous sucrose density gradient at neutral pH

A431 post-nuclear supernatants were fractionated on a continuous sucrose gradient. Fractions were assayed for (a) PI4K activity using endogenous PI as substrate and (b) PI4KII β , PI4KII α , VCP, PIPK1 α and PTP α by Western blotting. (c) The specificity of purified polyclonal antisera against PI4KII α and PI4KII β was tested using Western blotting against equal amounts of recombinant GST, GST-PI4KII α and GST-PI4KII β .

present in the higher-density fractions [9]. Before characterizing the enzymic activity of the two apparent pools of PI4KII α , it was therefore necessary to remove the contribution made by type III PI4K activity.

To investigate the contribution of PI4KII β to the measured PI4K activity an antiserum was raised against recombinant protein. Western blotting demonstrated that this reagent recognized recombinant PI4KII β , but not PI4KII α (Figure 1c). Subsequent blotting of A431 fractions prepared using continuous gradient ultracentrifugation revealed the presence of PI4KII β in a high-density region of the gradient (peak in fraction 12), largely separated from the peak of PI4KII α activity in fractions 9–10 (see also Figure 1a in [9]) and PI4KII α protein in fractions 9–12. The region of the gradient containing PI4KII β has been shown to be rich in ER and plasma membrane markers [9], consistent with the previously reported localization of activated PI4KII β to the plasma membrane of HeLa and COS-7 cells [8]. The presence of

PI4KII β in fractions 11–13 indicated that this isozyme contributed to the apparent PI4K activity of the higher-density pool of PI4KII α and would have to be removed before any kinetic analysis could be performed.

PITP α activity has been implicated in the provision of the PI substrate to PI4KII during receptor signalling [14] and may therefore also affect the activity measured across the density gradient. Indeed, previous results demonstrated that PITP enhances PI4K activity in purified caveolae [10]. Consequently an initial analysis used Western blotting to identify the presence of this protein in each fraction. Western blotting revealed that PITP α was clearly separated from all PI4K activity within the gradient (Figure 1b). Therefore, at least in unstimulated A431 cells, PITP was not required for the observed pattern of PI4K activity.

In order to explore whether or not either pool of PI4KII α corresponded with the reported agonist-sensitive activity [15,16], the location within the gradient of the agonist-sensitive PIPKII α isozyme [17,18] and the effects of known agonists were investigated. The distribution of PIPKII α overlapped with the less active pool of PI4KII α (Figure 1b), indicating that this pool contained the agonist-sensitive PI4KII α . However, addition of epidermal growth factor or bradykinin to serum-starved A431 cells did not result in any reproducible increase in PI4P production across the density gradient, nor any redistribution of PI4KII α , PITP or PIPKII α (results not shown). Although the two pools of activity observed in this gradient did not therefore appear to correspond to agonist-sensitive pools, the gradient fractionation procedure necessarily entails a separation of cytosol and membranes over a time course of several hours. Consequently these results do not exclude the importance of dynamic and reversible translocations of proteins such as PITP in agonist-stimulated PI4P production [19,20].

Having established the presence of other PI kinases in the PI4KII α -containing gradient fractions, it was necessary to improve the purity of the membrane preparation. The incorporation of sonication rather than mild homogenization, a discontinuous rather than a continuous sucrose gradient, and sodium carbonate buffer at pH 11 instead of a neutral buffer were investigated. A continuous gradient in high-pH buffer has been widely used following sonication to separate low- from high-buoyant-density membranes and had been used previously to prepare low-buoyant-density membranes (fractions 4 and 5) containing PI4KII α [10]. A major advantage of using sonication in sodium carbonate buffer was the dissociation of peripheral membrane proteins, including PIPK and type III PI4K enzymes. Indeed, amongst all the known PI kinases, phospholipases and transfer proteins only acylated PI4KII α and PI4KII β would be expected to remain attached to membranes under these conditions. Nonetheless, the low-buoyant-density fractions (4 and 5) in the previously employed discontinuous gradient were heterogeneous and included fragments derived from the plasma membrane and the ER, containing EGFR and calnexin, respectively ([21] and M. G. Waugh and J. J. Hsuan, unpublished work). Consequently differential solubility in various detergents was investigated as a means to selectively solubilize interfering membranes while leaving the PI4P-generating membrane fragments intact. Precedents for this approach came from work over the last decade on the isolation of detergent-insoluble, cholesterol-rich microdomains (rafts) of the plasma membrane, where non-ionic detergents such as Triton X-100 and Lubrol WX had been used to isolate caveolae and non-caveolar membrane rafts [22,23]. Such selective solubilization conditions remove most membranes completely to the soluble phase and also improve the resolution afforded by density gradient centrifugation by producing more homogeneous populations containing only detergent-insoluble microdomains. One of the

Figure 2 Subcellular fractionation of sonicated A431 cells on a discontinuous sucrose density gradient in the presence of detergent at pH 11

A431 cells were sonicated in buffer containing β -OG/DOC and sodium carbonate, pH 11, and fractionated on a discontinuous sucrose gradient. Fractions were assayed for (a) EGFR, calnexin and PI4KII α by Western blotting, (b) PI4K activity using either 200 μ M PI (●) or endogenous PI substrate (■), and (c) 3 H-labelled PI (□) or PI4P (○).

most commonly used detergents in the isolation of membrane microdomains is Triton X-100, which is normally used at a concentration of 1% (v/v). However, trial experiments demonstrated that Triton X-100 was effective at solubilizing PI4KII α at concentrations as low as 0.1% v/v (results not shown). An alternative detergent was therefore required to solubilize contaminating membranes, but not PI4KII α -containing membranes. A solution was found to be a combination of 10 mM β -OG and 4 mM DOC (β -OG/DOC), both used below their critical micelle concentration. Solubilization of buoyant plasma membrane by β -OG and ER fragments by DOC, both used above their critical micelle concentration, have been reported previously (see for example [22,24,25]). Serendipitously, PI4KII α -containing membranes proved insoluble in this detergent mixture. The established discontinuous gradient procedure was therefore modified by including β -OG/DOC in the cell-disruption buffer in order to remove contaminating plasma membrane and ER membranes. Analysis of the resulting gradient (Figure 2a) revealed as expected that the presence of β -OG/DOC removed EGFR and calne-

Figure 3 PI4KII β does not account for the major difference in activity between buoyant and dense membranes in the discontinuous gradient

(a) The distribution of PI4KII β in the discontinuous gradient was determined by Western blotting using purified anti-PI4KII β antiserum. (b) PI4KII α activity was assayed following immunoprecipitation from Triton X-100-solubilized and cleared buoyant membranes (fractions 4 and 5, ●) and dense membranes (fractions 11 and 12, ■) using purified anti-PI4KII α antiserum.

xin to fractions 10–12, which contained soluble proteins and high-buoyant-density membranes. In contrast, the addition of β -OG/DOC did not affect the distribution of PI4KII α activity which remained at the 5–35% sucrose interface (fractions 4 and 5 in Figure 2b). Furthermore, a clear separation of different pools of PI4KII α was revealed by Western blotting: most of the activity was present in fractions 4–5, while most of the PI4KII α protein co-localized with the plasma membrane and ER markers, EGFR and calnexin respectively, in fractions 10–12 (Figure 2a).

In order to test whether the distribution of PI4K activity measured using endogenous substrate could be affected by an uneven distribution of the substrate itself, the amount of PI in each fraction was determined using equilibrium metabolic labelling with [3 H]inositol. The results demonstrated that the bulk of the cellular phosphoinositides were not solubilized by β -OG/DOC and co-fractionated with the PI4K activity in low-buoyant-density membranes (Figure 2c). Nonetheless, approx. 2.5% of the endogenous PI was present in fractions 9–12 and PI4P synthesis was qualitatively unaffected by the addition of excess exogenous PI substrate (Figure 2b).

A final question concerning the apparent PI4K activity in this gradient was the potential contribution made by PI4KII β . Western blotting revealed that the bulk of the detectable PI4KII β was found in the bottom of the 45% sucrose region of the gradient (fraction 12), which contained soluble proteins. Dissociation of PI4KII β but not PI4KII α from membranes at high pH is consistent with the ability of PI4KII β but not PI4KII α to translocate between cytosol and membrane [8]. EGFR and calnexin are both transmembrane proteins. As the distribution of PI4KII β did not follow either peak of PI4K activity, it was inferred that both peaks were predominantly due to PI4KII α activity (Figure 3a). To further establish that the high and low peaks of PI4K activity arose from differences in the activity of the PI4KII α isozyme, the

Scheme 1 Procedure for the separation and analysis of buoyant and dense membranes containing PI4KII α

rates of PI4P production were compared using PI4KII α immunoprecipitated from each pool using specific antibodies. Similar proportions (approx. 1 %) of the total activity in each pool could be immunoprecipitated. As can be seen in Figure 3(b), PI4KII α immunoprecipitated from the buoyant fraction had a much higher activity relative to PI4KII α immunoprecipitated from the dense fraction, thereby confirming a difference of intrinsic activity between the two pools of PI4KII α .

These results therefore confirmed the existence of differentially active forms of PI4KII α and that the combination of sonication in β -OG/DOC and sucrose density centrifugation was sufficient to clearly separate membrane fragments containing these two forms. Furthermore β -OG/DOC removed plasma membrane and ER contaminants from fractions 4–5, thereby improving the purification of the low-buoyant-density, PI4KII α -containing membranes. Although solubilized proteins co-fractionated with the higher-buoyant-density PI4KII α -containing membranes (fractions 10–12), they were easily removed by subsequent centrifugation for 45 min at 100 000 *g*. In addition to removing soluble proteins, this latter ultracentrifugation step served to remove sucrose, carbonate and β -OG/DOC from both pools of activity. The total procedure is summarized in Scheme 1.

As Michaelis–Menten analysis requires that the enzyme of interest is freely diffusible, β -OG/DOC-insoluble, 100 000 *g* pellets were dissolved in $2 \times$ PI4K assay buffer, which contained 0.8 % Triton X-100, and cleared prior to kinetic analyses. The absence of PI4KIII isozymes was confirmed using Western blotting (Figure 4a). At this stage of the analysis it was possible that the apparent differences in PI4K activity between the buoyant and dense pools could have been due to heterologous, membrane-

Figure 4 Analysis of PI4P synthesis by purified buoyant and dense membranes

The two purified pools of PI4KII α -containing membranes were solubilized in Triton X-100 and cleared. (a) Absence of all detectable PI4KIII α and β isozymes from the Triton X-100 supernatant of fractions 11 and 12 (lane 1) and their removal to the corresponding supernatant (lane 2) was tested by Western blotting equivalent volumes with isozyme-specific antibodies. (b) The presence of PI4K-stimulating or -inhibiting activities in the purified buoyant and dense membranes was tested. Membranes from each pool were solubilized in Triton X-100 and cleared. The PI4K activity of individual and mixed supernatants was then compared with the predicted activity. (c) PI4K activity of Triton X-100-solubilized and cleared buoyant (●) and dense (■) fractions was assayed using different concentrations of exogenous PI.

bound activators or inhibitors, respectively. For example, heterotrimeric G-proteins, small G-proteins and phospholipids have been reported to modulate type II PI4K activity [8,26,27]. To test these concerns, an experiment was performed which involved mixing equal aliquots of purified, solubilized PI4KII α from each pool in order to test whether or not the amount of PI4P produced would be simply additive. The results of this experiment clearly showed that the PI4K activity produced by mixing different pools was additive (Figure 4b), which indicated that the difference in activity between the two pools was not caused by heterologous activators or inhibitors.

To characterize the apparently intrinsic differences between the two pools of PI4KII α activity, their $V_{\max}$ and $K_m(\text{PI})$ were compared (Figure 4c). The $V_{\max}$ of the more buoyant membranes was approx. 3.5-fold higher than the $V_{\max}$ of the denser membranes (0.39 ± 0.02 and 0.11 ± 0.03 fmol of PI4P/min, respectively, $P < 0.01$, $n = 3$). In addition, an approx. 4-fold difference in $K_m(\text{PI})$ was apparent between the samples (19.8 ± 7.1 and 81.9 ± 17.6 μM respectively, $P < 0.05$, $n = 3$). These results revealed that an increase in $V_{\max}$ and a decrease in $K_m(\text{PI})$ of

the PI4KII α in the buoyant relative to the denser membranes is the reason underlying the different rates of PI4P synthesis observed in the gradient fractions, and confirm the existence of differentially active pools of PI4KII α in unstimulated A431 cells.

DISCUSSION

In these studies we demonstrate that cellular PI4P production is largely accounted for by two distinct pools of PI4KII α activity, both of which can be isolated as β -OG/DOC-insoluble membranes. A highly active PI4KII α pool is of low buoyant density, while a less active form of the enzyme is of higher buoyant density. It is noteworthy that both of these membrane pools are readily soluble in Triton X-100 and are therefore not equivalent to the various Triton X-100-insoluble rafts that have been extensively studied in recent years. Instead the β -OG/DOC-resistant membranes identified here seem to represent a novel subtype of membrane microdomain. The observation that the buoyant and dense pools of PI4KII α both localize to β -OG/DOC-insoluble membranes suggests that similar PI4KII α -containing membrane microdomains may exist in many of the organelles to which PI4KII α has been localized, such as the Golgi [8] and endosomal compartments [28]. Although the higher activity pool may be derived from a number of different cellular membranes, the analysis reported here is consistent with their identification as the low-buoyant-density, ER-related PI4KII α activity, previously found to be distinct from the plasma membrane, ER and *trans*-Golgi network.

Importantly, numerous previous studies have failed to distinguish between different pools of PI4K activity, for example during phosphoinositide 3-kinase and phospholipase C signalling. The previously unsuspected existence of membranes containing differentially active forms of PI4KII α in unstimulated cells and the occurrence of this enzyme in many different subcellular membranes raise the need to reconsider the significance of small changes in total PI4K activity observed for example during receptor signalling (e.g. see [16,29]). If these small changes are localized to a small pool of low-activity enzyme, then the magnitude of activation will be far greater than previously inferred.

The difference in activity between the buoyant and dense pools is due to a 3.5-fold increase in $V_{\max}$ and a 4-fold decrease in K_m (PI) in the buoyant fraction. The K_m (PI) values obtained here fall within the range of previously reported values for PI4KII α , which are characteristically lower than type III isozymes [3]. The constitutively enhanced and agonist-insensitive PI4K activity in the buoyant fraction described here appears to differ from epidermal growth factor-stimulated PI4KII activity in A431 cells [15,16] where epidermal growth factor induced a decrease in K_m (PI) from 20 to 8 μ M with no change in $V_{\max}$ [15]. This difference in enzymic properties indicates that distinct mechanisms of activation exist for agonist-stimulated and constitutively enhanced PI4P synthesis. Future studies will address the molecular basis of these different mechanisms of activation.

Finally, it is interesting to consider the possible functional importance of differentially active pools of PI4KII α . The results using equilibrium 3 H-labelling of phosphoinositides indicate that the buoyant membrane fraction is the major site of PI4P accumulation as well as its synthesis, and that the denser PI4KII α -containing membranes contain very little PI4P. Why should the PI4KII α isozyme be the only known PI kinase to exist in two different states of activity in unstimulated cells? The first point to make is that PI4KII α is the only PI kinase that is constitutively membrane-bound. All other PI kinases are soluble and appear to be activated by mechanisms involving recruitment to target membranes. Consequently just as the type II α isozyme is often

assumed to be constitutively active, PI4P has been assumed to be freely available as a substrate for PI4P 3- and 5-kinases and to have no signalling role of its own. The results from this study demonstrate that most PI4KII α is in fact of relatively low activity, while most of the measurable PI4KII α activity and PI4P are localized to a minor membrane fraction. Furthermore, recent results suggest that PI4P does have a signalling role: for example PI4P-rich membranes may recruit PI4P-specific binding proteins, such as OSBP (oxysterol-binding protein), FAPP1 (PI4P adaptor protein 1) or EpsinR [4–6]. In addition a high content of PI4P may bring about physical changes in the buoyant membrane domain, such as altered charge and curvature. The precise role of PI4P in the buoyant membranes and whether PI4KII α in the denser membranes can be stimulated are areas of ongoing study.

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