Localization of a highly active pool of type II phosphatidylinositol 4-kinase in a p97/valosin-containing-protein-rich fraction of the endoplasmic reticulum

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Different phosphoinositides are synthesized in cell membranes in order to perform a variety of functions. One of the most abundant of these lipids is phosphatidylinositol (PI) 4-phosphate (PI4P), which is formed in human eukaryotes by type II and type III phosphatidylinositol 4-kinase (PI4K II and III) activities. PI4K II activity occurs in many different subcellular membranes, although no detailed analysis of the distribution of this activity has been reported. Using density gradient ultracentrifugation, we have previously found that in A431 cells the predominant PI4K activity arises from a type IIα enzyme that is localized to a buoyant membrane fraction of unknown origin [Waugh, Lawson, Tan and Hsuan (1998) J. Biol. Chem. **273**, 17115–17121]. We show here that these buoyant membranes contain an activated form of PI4K IIα that can be separated from the bulk of the PI4K IIα protein in A431 and COS-7 cells. Proteomic analysis revealed that the buoyant membrane fraction contains numerous endoplasmic reticulum (ER)-marker proteins, although it was separated from the bulk of the ER, ER–Golgi intermediate compartment, transitional ER, Golgi and other major subcellular membranes. Furthermore, the majority of the cytoplasmic valosin-containing protein (VCP), an AAA+ ATPase implicated in various ER, transitional ER, Golgi and nuclear functions, was almost completely localized to the same buoyant membrane fraction. Co-localization of VCP and PI4K activity was confirmed by co-immunoprecipitation. These results suggest the previously unsuspected existence of an ER-related domain in which the bulk of the cellular PI4P synthesis and VCP are localized.

Key words: endoplasmic reticulum, phosphatidylinositol kinase, phosphoinositide signalling, subcellular fractionation, valosin-containing protein.

INTRODUCTION

Phosphatidylinositol 4-phosphate (PI4P) is a phospholipid of central importance in many cellular processes. In addition to the role of PI4P as a substrate precursor for phospholipase C, phosphoinositide 3-kinase and other signalling pathways, PI4P may have biological activity in its own right; for example, in the binding and targeting of proteins containing a PI4P-specific pleckstrin homology domain to particular subcellular membranes [1]. Two main classes of enzymes have been discovered that phosphorylate PI to form PI4P: type II, characterized by their sensitivity to inhibition by micromolar concentrations of adenosine and by the 4C5G monoclonal antibody, and type III, which are more closely related to the phosphoinositide 3-kinase family and are inhibited by wortmannin (reviewed in [2,3]). Each of these types comprises two distinct forms in mammalian cells, termed α and β. Measurements of PI4P synthesis in mammalian cells have indicated that type II activity is predominant in various non-neuronal cell types; in such cells, PI 4-kinase (PI4K) II activity has been reported to localize mainly to the plasma membrane [4–6] and lysosomes [4,7]. Additionally, PI4K II activity has been found associated with secretory vesicles [8], coated vesicles [9], GLUT4 transport vesicles [10] and small synaptic vesicles [11]. This contrasts with the situation in yeast, where most of the measurable PI4K activity can be accounted for by the activities of the yeast PI4K III homologues pik1 and stt4, which function at the Golgi [12] and plasma membrane [13] respectively. The yeast PI4K II homologue, Lsb6, has recently been cloned [14] and, unlike PI4K III homologues [15], its deletion does not give rise to any apparent phenotype, suggesting that it may have a more subtle or highly regulated role than the predominant PI4K III enzymes. Taken together, these data suggest that PI4K II and III isoenzymes are widely distributed among the cytosol and various subcellular membranes in mammalian cells. However, in homogenized A431 cells the predominant PI4K IIα activity is highly localized to low-buoyant-density membranes following subcellular fractionation on sucrose gradients [16].

Recently, the molecular cloning of mammalian PI4K IIα and IIβ [17–19] has permitted the use of immunological methods for the detection of both endogenous and recombinant enzymes in mammalian cells. This development means that measurements of PI4P production in membranes from fractionated cells can now be investigated in parallel with the distribution of the different PI4K isoenzymes. Initially, it was demonstrated that PI4K IIα and IIβ were both localized mainly to endosomal vesicular structures [19]. However, this finding has been challenged in a recent report, where the α isoform was observed to mainly associate with the Golgi, and, more controversially, the β isoform was cytosolic, but recruited to the plasma membrane by rac1 [20].

Abbreviations used: EEA1, early endosome antigen 1; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERGIC, ER–Golgi intermediate compartment; ESI, electrospray ionization; ESI-MS/MS, tandem ESI MS; LC, liquid chromatography; PI, phosphatidylinositol; PI4K, PI 4-kinase; PI4P, PI 4-phosphate; PNS, post-nuclear supernatants; tER, transitional ER; TGN, *trans*-Golgi network; VCP, valosin-containing protein.

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In the present paper we present a rigorous analysis of the subcellular origin of the main peak of PI4K IIα activity in A431 cells. We demonstrate through a combination of cell fractionation, proteomics and confocal microscopy that it is mainly associated with previously unidentified p97 valosin-containing protein (VCP)-rich membranes. These membranes appear to be related to the endoplasmic reticulum (ER), but are distinct from the bulk of the ER, COPII-containing ER exit sites in the transitional ER (tER), the ER–Golgi intermediate compartment (ERGIC) and Golgi membranes. These results reveal that, at least in some cell types, most PI4P biosynthesis may be confined to a VCP-rich intracellular domain.

EXPERIMENTAL

Materials

Anti-(PI4K IIα) serum was raised in New Zealand White rabbits using recombinant PI4K IIα, and was shown to specifically recognize this isoform. Anti-epidermal growth factor receptor ('EGFR') peptide 2E antiserum has been described previously [21]. 4C5G and antibodies against p230, ERGIC-53, mSEC13p and stomatin and were kindly provided by Dr J. Backer (Department of Molecular Pharmacology, Albert Einstein College of Medicine, New York, NY, U.S.A.), Dr G. Endemann (Department of Molecular Pharmacology, Stanford University Medical School, Stanford, CA, U.S.A.), Dr H.-P. Hauri (Biozentrum, University of Basel, Basel, Switzerland), Dr B. L. Tang (Institute of Molecular Cell Biology, The National University of Singapore) and Dr G. Stewart (Department of Medicine, University College London, London, U.K.) respectively. Antibodies against syntaxin 4, syntaxin 6, prenylcysteine lyase, p115, annexin II and early endosome antigen 1 (EEA1) were from BD Biosciences Pharmingen (San Diego, CA, U.S.A.). Anti-calnexin and anti-p92 antibodies were from Stressgen and Santa Cruz respectively. Secondary antibodies for Western blotting, ECL® reagents and radiolabelled reagents were from Amersham Biosciences. Cell-culture reagents were from Life Technologies. Protein assay reagents were purchased from Bio-Rad. Protease-inhibitor cocktail tablets (Complete™) were from Roche Diagnostics. All other reagents were obtained from Sigma.

Cell culture and metabolic radiolabelling

A431 and COS-7 cells were maintained at 37 °C in a humidified incubator at 10 % CO_2. Cells were cultured in Dulbecco's modified Eagle's medium ('DMEM') containing Glutamax, 10 % (v/v) fetal-calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin. Metabolic radiolabelling of phosphoinositides was performed using [^{3}H]inositol, as described previously [16].

Subcellular fractionation of cells

Confluent monolayers were placed on ice, washed twice with ice-cold PBS, pH 7.4, and 10 mM Tris/HCl, pH 7.4, was added for 1 min. Cells were scraped into homogenization buffer comprising 10 mM Tris/HCl, 1 mM EGTA, 0.5 mM EDTA and 0.25 M sucrose, pH 7.4, also containing Complete™ protease inhibitors, and homogenized with ten strokes of a loose-fitting Dounce homogenizer. All subsequent steps were carried out at 4 °C. Post-nuclear supernatants (PNS) were obtained by centrifugation for 10 min at 1000 ***g***. PNS (2 ml) were layered on to a sucrose gradient [successive layers of 1 ml of 40 % (w/v), 1 ml of 30 %, 2 ml of 25 %, 2 ml of 20 %, 2 ml of 15 % and 2 ml of 10 % sucrose]. Gradients were centrifuged for at least 16 h at 175 000 ***g***. After centrifugation, 12 1-ml fractions were harvested, beginning at the top of the gradient. The pellet was resuspended in 1 ml of 0.25 M sucrose, and designated fraction P (or 13).

MS analysis of PI4K-rich domains from A431 cells

The peak PI4K activity fraction from A431 cells (see Figure 1) was adjusted to 0.1 M Na_2CO_3, sonicated, adjusted to 45 % (w/v) sucrose, and placed at the bottom of a discontinuous sucrose gradient consisting of 4 ml of 45 % sucrose, 4 ml of 35 % sucrose and 4 ml of 5 % sucrose in 10 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM EDTA, pH 7.4. The gradient was centrifuged overnight at 4 °C at 175 000 ***g***. PI4K activity was concentrated at the interface of the 35 and 5 % sucrose layers, as reported previously [16]. Membranes from this fraction were resuspended in 0.1 M Tris/HCl, pH 8.0/20 mM NaCl/1 mM dithiothreitol containing 100 mM β-octyl glucoside and 40 mM deoxycholate, and cleared by centrifugation for 30 min at 18 000 ***g***. The supernatant was fractionated using a MonoQ Smart column (Amersham Biosciences) developed with a discontinuous gradient to 1 M NaCl, as described previously [17]. Proteins in each fraction were separated further by SDS/PAGE and analysed by tandem electrospray ionization (ESI) MS (ESI-MS/MS), as described previously [17].

Immunoblotting

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

Co-immunoprecipitation of PI4K IIα and VCP

Aliquots (100 μl) of the peak PI4K activity fraction prepared from A431 cells were pre-incubated for 45 min with 10 μl of Protein A–Sepharose (Amersham Biosciences). The Protein A beads and non-specifically bound material were removed by centrifugation, and the supernatant was incubated for 1 h at 4 °C with 15 μl of either anti-(PI4K IIα) serum or matched pre-immune serum. Immune complexes were adsorbed on to 10 μl of Protein A beads by a further incubation for 45 min at 4 °C, before washing 4 times in 500 μl of homogenization buffer without sucrose. Samples were analysed by SDS/PAGE and Western blotting with anti-VCP antibody, and by assaying PI4K activity.

PI4K assays

Assays in the presence of endogenous and exogenously added PI were performed as described previously [16].

Scanning confocal microscopy

A431 and COS-7 cells were grown on glass coverslips for at least 48 h before fixation and permeabilization, as described in [22]. Coverslips were stained with the appropriate primary antibody, followed by highly cross-adsorbed goat anti-rabbit IgG linked to Alexa Fluor 488, and goat anti-mouse IgG linked to Alexa Fluor 568 (Molecular Probes, Eugene, OR, U.S.A.). Confocal immunofluorescence images were collected using a

Bio-Rad Radiance 2100 system in conjunction with a Nikon Eclipse E800 microscope (kindly provided by Dr T. Cowen, Department of Anatomy and Developmental Biology, University College London, London, U.K.). All images were collected as single confocal sections using separate excitation with blue (488 nm) or yellow (568 nm) laser lines (krypton-argon mixed gas laser) and Lasersharp 2000 software. Pinhole diameters were set at each wavelength to ensure that confocal sections of equal depth were collected in each channel.

RESULTS

Previous reports demonstrated that PI4K IIα activity is predominant in A431 human epidermoid carcinoma cells, where it is concentrated in buoyant, non-caveolar membranes located at the interface of 5 and 35 % sucrose in a discontinuous gradient [16]. We sought to define the subcellular origin of these membranes by separating membranes cleared of nuclei on a continuous 10–40 % sucrose gradient, designed to separate buoyant membranes. Fractions of the gradient were analysed by Western blotting with a large panel of organelle-specific marker proteins and by assaying PI4P synthesis using the incorporation of [^{32}P]P_i from radiolabelled ATP into endogenous PI. The distribution of endogenous PI was revealed using metabolic labelling with [^{3}H]inositol and found to peak in fraction 12, although PI was present in fractions 8–13 (Figure 1a). A single major peak of PI4K activity was found at fraction 9 and a shoulder at fractions 10–11 (Figure 1a), a distribution that was little affected by addition of either detergent or additional PI to the assays. Unexpectedly, however, the distribution of this peak did not coincide with marker proteins for the plasma membrane, Golgi, *trans*-Golgi network (TGN), ER, tER, ERGIC, exocytic vesicles, endosomes or lysosomes (Figure 1b). This distribution of PI4K activity was not confined to A431 cells, since very similar distributions were observed using COS-7 simian kidney and HepG2 human liver cell lines (results not shown).

The activity in fraction 9 was confirmed as type IIα by its separation from type III enzymes, inhibition with monoclonal antibody 4C5G (results not shown), identification by MS of PI4K IIα in fraction 9 (see below), and immunoprecipitation by anti-(PI4K IIα) serum (also see below). Immunoblotting with an antiserum raised against the PI4K IIβ isoform revealed barely detectable levels of this enzyme in A431 cells compared with the α isoform (S. Minogue, M. G. Waugh and J. J. Hsuan, unpublished work). Furthermore, separation of the peak activity from the bulk of PI4K IIα protein detected by immunoblotting (Figure 1a) indicates that the activity measured in fraction 9 arises from an activated form of the enzyme.

To characterize further the membranes containing activated PI4K IIα in different cell types, their protein composition was examined. A431 membranes from the peak PI4K activity fraction were stripped and sonicated in 0.1 M Na_2CO_3 (pH 11) in order to reduce complexity and bias the analysis towards resident membrane proteins, and purified further on a discontinuous sucrose-density gradient. Proteins remaining in the purified membranes were solubilized and separated using ion-exchange chromatography and SDS/PAGE, before identification by ESI-MS/MS. Eighty-nine proteins were identified (results not shown), including PI4K IIα and the PI4P-specific 4-phosphatase mSAC1p, which is localized to the ER [23]. Of those 89 proteins, 46 are known to have a predominantly single subcellular localization (Table 1), and appeared to be derived from five organelles: ER (72 %), TGN (9 %), plasma membrane (4 %), endosomes (11 %) and mitochondria (4 %). The vast majority of the membranes

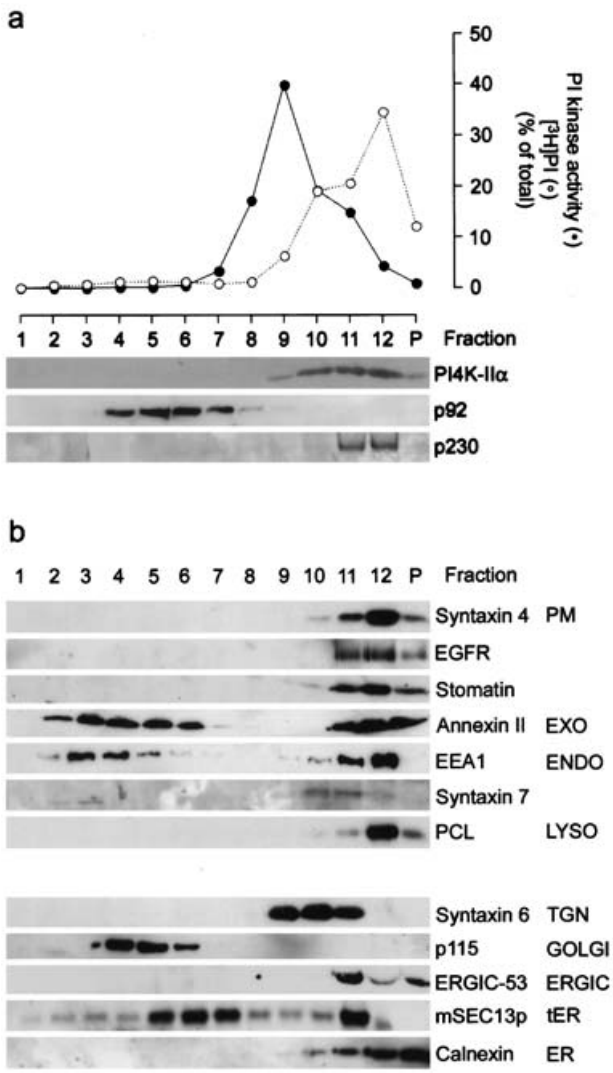

Figure 1 Subcellular fractionation of A431 cell PNS

PNS prepared from A431, COS-7 and HepG2 cells were separated on a 10–40 % sucrose gradient, and 1 ml gradient fractions were analysed as follows: (**a**) PI4K activity (black circles) and [^{3}H]PI (white circles) distributions were measured across the gradient and compared with Western blots for PI4K IIα and type III PI4Ks (p230/α and p92/β). There was too little PI4K IIβ to detect in these preparations. (**b**) Parallel Western blots for organelle marker proteins (EXO, exocytotic vesicles; ENDO, endosomes; LYSO, lysosomes). Results are shown from A431 cell preparations only, since similar activity profiles were obtained using COS-7 and HepG2 cells. PCL, prenylcysteine lyase.

in fraction 9 were therefore derived from the ER, despite the fact that most of the ER-marker, calnexin, was in fractions 11–13 (Figure 1b). The presence of a few Golgi and endosomal proteins was probably due to the spread of membranes that peaked in neighbouring fractions: peak immunoreactivity to syntaxin 6 (a TGN and TGN-derived vesicle protein) and syntaxin 7 (an early and late endosomal protein) appeared in fractions 10 and 11 (Figure 1b). In addition, transmembrane proteins from all organelles are first inserted in the ER membrane during biosynthesis. Finally, although a few mitochondrial proteins were also present, removing mitochondria by centrifugation did not remove the peak of PI4K activity (results not shown).

To confirm that the membranes rich in PI4K activity were derived from the ER, membrane and peripheral proteins in each of the fractions described in Figure 1 prepared from COS-7

Table 1 Identification of marker proteins in peak PI4K activity fraction

The peak PI4K activity fraction from A431 cells (see Figure 1) was stripped of peripheral proteins and purified further using a discontinuous sucrose gradient. Membranes in the fraction containing the peak of PI4K activity were solubilized in detergent and fractionated using anion-exchange chromatography as described previously [17]. Proteins in each column fraction were separated further by SDS/PAGE and identified by ESI-MS/MS. PI4K IIα was identified in this analysis, but is not included as it localizes to multiple subcellular organelles (PM, plasma membrane; G, Golgi; E, endosomes; M, mitochondria). GPI, glycosylphosphatidylinositol; NSF, *N*-ethylmaleimide-sensitive fusion protein.

No.	Protein	Organelle
1	ATPase, Ca^{2+}-transporting	ER
2	Nogo-A	ER
3	Integrin α6	PM
4	Heat-shock protein gp96	ER
5	α-Glucosidase II	ER
6	Calnexin	ER
7	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase	ER
8	Plakoglobin	PM
9	Glucosidase II, β subunit	ER
10	Heat-shock 70 kDa protein 5	ER
11	Cytochrome P450 reductase	ER
12	Ribophorin I	ER
13	Heat shock 70 kDa protein 1B	ER
14	Ribophorin II	ER
15	UDP-GalNAc transferase 2	G
16	ER-63; similar to cytoskeleton-associated protein 4	ER
17	Prolyl 4-hydroxylase	ER
18	Suppressor of actin 1 (mSAC1p)	ER
19	ER-60 protease, ERp57, GRP58	ER
20	UDP-glucuronosyltransferase 1A10	ER
21	Cytochrome P450, CYP1B1	ER
22	Fatty aldehyde dehydrogenase ('FALDH')	ER
23	Cytochrome P450, CYP51 (lanosterol 14α-demethylase)	ER
24	ETEA	ER
25	UDP-glucuronosyltransferase 1A6	ER
26	Serine palmitoyltransferase	ER
27	Calreticulin	ER
28	Zinc metalloproteinase, STE24p homologue	ER
29	Nogo-B	ER
30	GPI transamidase (gpi8)	ER
31	Probable syntaxin 12	E
32	Syntaxin 7	E
33	Syntaxin 10	G
34	Androgen-regulated short-chain dehydrogenase/reductase 1	ER
35	Catechol-O-methyltransferase ('COMT') S- or MB-isoform	ER
36	Dolichyl-phosphate-mannosyltransferase polypeptide 1	ER
37	RAB12	G
38	RAB32	M
39	Vesicle-associated soluble NSF-attachment-protein-receptor v-SN	G
40	ADP, ATP carrier protein T2	M
41	BAP31	ER
42	RAB5B	E
43	RAB5C	E
44	Type I receptor isoform	ER
45	RAB7	E
46	Mitsugumin23	ER

cells were separated using SDS/PAGE in order to identify any proteins that paralleled the distribution of PI4K activity. Only a few such proteins were apparent. Of these, one was relatively abundant and successfully identified by ESI-MS/MS as VCP (also termed p97) (Figure 2a). VCP is a multi-ubiquitin-binding AAA+ ATPase localized predominantly to the ER and nucleus [24,25], where it has key roles in ER-associated protein degradation (ERAD) [26–28], and probably nuclear transport [25], respectively. VCP has also been implicated in the fusion of tER [29,30] and in regenerating Golgi membranes following mitosis

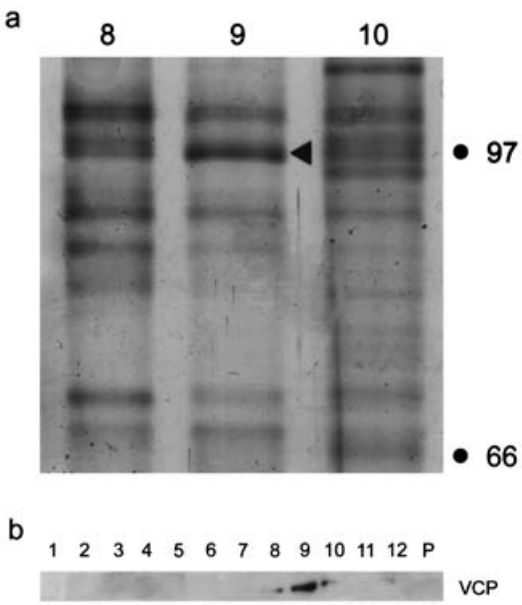

Figure 2 Identification and co-fractionation of VCP

Gradient fractions from A431 and COS-7 cells were prepared as described in Figure 1(a). Equal aliquots from gradient fractions 8–10 prepared from COS-7 cells were separated by SDS/PAGE and visualized by silver staining [39]. A protein at 97 kDa peaked in the PI4K-activity-rich fraction 9 (shown by the arrowhead). This band was excised and analysed by ESI-MS/MS, and found to be p97/VCP. (**b**) Equivalent aliquots from gradient fractions prepared from A431 cells were analysed by SDS/PAGE and Western blotting with anti-VCP antibodies. The positions of molecular-mass markers (in kDa) are shown to the right of the gel.

[31,32]. Consistent with the COS-7 cell analysis (Figure 2a) and the removal of nuclei prior to all gradient analyses, immunoblotting A431 cell preparations revealed the expected narrow localization of VCP in the density gradient (Figure 2b). This did not overlap with the ER-marker calnexin, but virtually all the VCP overlapped with the peak of PI4K activity (compare Figure 2b with Figures 1a and 1b). Consequently, the VCP/PI4K-containing membranes were tentatively inferred to comprise a discrete part of the ER, that is separate from the bulk of the ER, nuclear envelope, tER and ERGIC. As a peripheral-membrane protein, VCP is stripped away from the membranes whose protein composition is reported in Table 1. Co-localization of VCP and PI4K IIα activity within a single membrane was also tested using co-immunoprecipitation. Anti-VCP antibodies were unable to immunoprecipitate VCP using intact membranes. However, antibodies against PI4K IIα were able to bind 5–10% of the PI4K activity in fraction 9 and, importantly, a similar proportion of VCP in fraction 9 prepared from A431 cells (Figure 3), thereby confirming their co-existence in a single membrane. No evidence for a direct interaction between VCP and PI4K IIα was obtained using immunoprecipitation from A431 cells solubilized in 1% (v/v) Triton X-100 (results not shown).

Confocal immunofluorescence microscopy was employed to compare the distribution of endogenous proteins in intact cells with the inferences drawn above. Although there are no reagents to unambiguously identify the small pool of activated PI4K IIα, it was important to confirm the separation of the VCP-rich membranes containing this active pool from endosomal and Golgi compartments, where most PI4K IIα protein is reportedly localized [19,20]. In COS-7 (Figures 4a, 4d and 4g) and A431 (not shown) cells, PI4K IIα antibodies produced a complicated

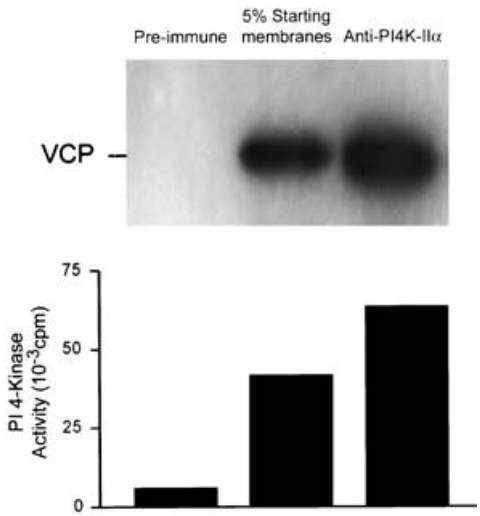

Figure 3 Co-immunoprecipitation of VCP and PI4K activity using intact membranes

Aliquots (50 μl; 5 %) of the peak PI4K activity/VCP fraction prepared from A431 cells were incubated with either matched pre-immune serum or anti-(PI4K IIα) serum, and adsorbed on to immobilized Protein A. An equivalent aliquot of the density-gradient fraction (5 % Starting membranes) and immunoprecipitated samples were analysed in parallel by SDS/PAGE and Western blotting with anti-VCP antibody (upper panel), and by assaying PI4K activity (lower panel).

pattern: there was clear staining of the nucleus, as well as a reticular cytoplasmic network and a juxtanuclear region. VCP was virtually completely localized to the nucleus and a similar juxtanuclear region (Figure 4b). The VCP-rich, juxtanuclear region presumably also contains the pool of activated PI4K IIα. However, as expected from the separation of VCP and PI4K IIα proteins (Figure 1a), on close inspection the juxtanuclear regions enriched in PI4K IIα and VCP do not coincide (Figure 4c).

In contrast with a previous study using overexpression of epitope- and green-fluorescence-protein-tagged PI4K IIα in COS-7 and HEK-293 cells [19], there was no clear co-localization of endogenous PI4K IIα with EEA1 in COS-7 (Figures 4d–4f) or A431 cells (results not shown). It remains possible that the endogenous PI4K IIα is present in endosomes, but is proportionally lower in abundance than that detected using ectopic overexpression of a fusion protein, as discussed in [20]. Overlap of PI4K IIα with the TGN marker syntaxin 6 (Figures 4g–4i) was more difficult to assess, because of the more complicated distribution of the former protein among several organelles. The reported presence of PI4K IIα in the Golgi of HeLa cells [20] is nonetheless supported by the overlapping patterns observed in COS-7 cells, and, to a lesser extent, in A431 cells (results not shown). However, there was no clear co-localization of PI4K IIα with syntaxin 6-positive TGN-derived vesicles [33], indicating enrichment of PI4K IIα protein only at the TGN in these cell types. Combined with the results from cell fractionation, these observations using confocal microscopy suggest that PI4P synthesis in VCP-enriched cytoplasmic membranes is due to a small pool of highly active PI4K IIα.

DISCUSSION

The results presented here open a new avenue in phosphoinositide research through the striking observation that the bulk of cellular PI4P production is confined to a VCP-enriched region related to the ER. The identification of a VCP-enriched ER domain that is distinct from the bulk ER, nuclear membrane and COPII-coated ER-export sites of the tER is also a novel and unexpected finding, and contributes to the emerging picture of specialization within the ER. The density-gradient profile of PI4P generation in the presence of exogenous substrate and activating concentrations of Triton X-100 was found to be similar to the distribution of activity when endogenous PI alone was the phospholipid substrate. Furthermore, the PI4P-specific phosphatase mSAC1p was present in the peak PI4K activity fraction. These observations suggest that, although the observed distributions of PI4K activity and endogenous PI differ, the former profile is unlikely to be due to varying levels of endogenous PI in the different membrane fractions or to the absence of a phosphatase in the peak fraction. Instead, it appears that the dominant PI4K activity, which co-localizes and co-immunoprecipitates with VCP, arises from a highly active pool of PI4K IIα.

These results do not contradict previously reported roles for PI4K IIα at subcellular localizations other than the VCP/PI4K domain. Indeed, we were able to show by confocal microscopy that endogenous PI4K IIα protein did partly overlap with the Golgi apparatus, as reported previously [20]. However, the present study underlines the value of analytical subcellular fractionation in parallel with confocal microscopy as a means of validating the subcellular localization of both an enzyme and its activity.

A possible interpretation of these results is that, at least in unstimulated cells, the specific roles of phosphoinositides in different organelles may require different threshold concentrations, which in turn arise through localizing PI4Ks of inherently different activity. Hence the various functions of PI4P may be executed at different concentrations in different areas of the cell. Furthermore, the present study supports the idea that more than one PI4K isoenzyme is present in some parts of the cell. For example, type II and III PI4Ks appear to be present at both the TGN and plasma membrane, and hence individual or combined activities may contribute at any one site towards the varied and dynamic roles that have been suggested for phosphoinositides. In line with this reasoning, it may be significant that the PI4K IIα isoform is the only known phosphoinositide kinase that is constitutively membrane-bound (by means of palmitoylation; [18]), and may therefore be the only phosphoinositide kinase with constitutive access to its lipid substrate. This suggests that, even in non-stimulated conditions, cells have a requirement for robust PI4K IIα-mediated PI4P production at the VCP-rich membranes.

Activation of PI4K IIα in the VCP-rich membranes identified here does not seem to be dependent on recruitment of a heterologous cytosolic factor, as occurs in the activation of PI4K IIIβ by neuronal calcium sensor-1 ('NCS-1'), since the PI4K IIα activity is preserved following treatment with carbonate buffer at pH 11.0. This suggests that protein or lipid components within the membrane itself and/or covalent modification of PI4K IIα may enhance PI4K activity in the VCP-rich membranes. Further studies are under way to define the mechanism of activation.

The co-localization of cellular PI4P synthesis and VCP in a subdomain of the ER is a novel finding, and may indicate the existence of some link between phosphoinositide metabolism and the functions that have been proposed for VCP. Since VCP has been implicated in ERAD, nuclear transport, fusion of the tER and post-mitotic Golgi reorganization, it is possible that the membrane fraction described here could be involved in one or more of these processes. A question immediately arises

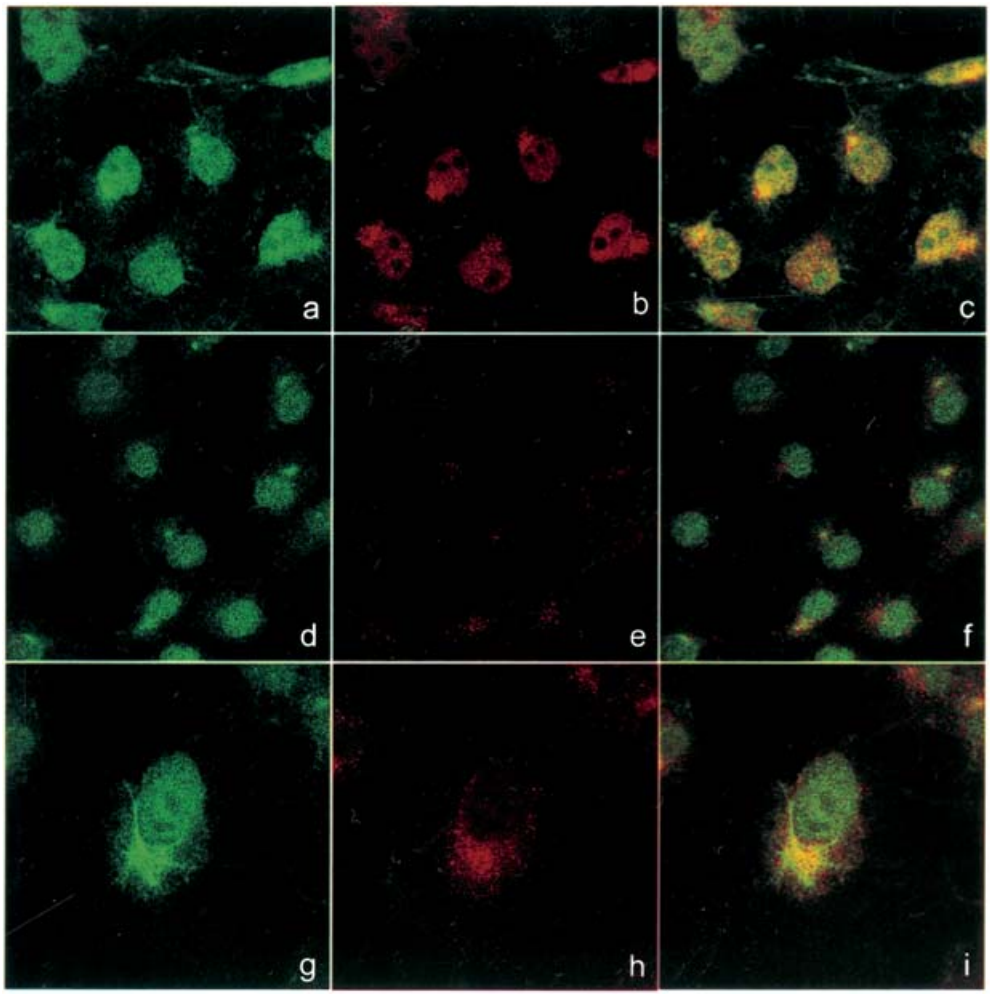

Figure 4 Localization of endogenous PI4K IIα, VCP, EEA1 and syntaxin 6 in COS-7 cells

COS-7 cells grown on glass coverslips were fixed and stained using antibodies against: PI4K IIα (**a**, **d**, **g**), VCP (**b**), EEA1 (**e**) and syntaxin 6 (**h**). The respective merged images are shown in panels (**c**), (**f**) and (**i**). Pre-adsorption of the anti-(PI4K IIα) serum with immobilized recombinant PI4K IIα [17] abolished staining (results not shown). The distribution of PI4K IIα revealed by these experiments was consistent with results from experiments in which A431 cells were separated by centrifugation into nuclear and PNS fractions, and then subjected to Western blotting with the anti-(PI4K IIα) antibodies (results not shown).

concerning the function that PI4K might have in any of these events. One possibility is the lateral organization and structure of membranes. In this regard, phosphoinositides have previously been localized to subdomains within the plasma membrane, Golgi and trafficking vesicles [34], and the ER appears to be divided into functional regions, many of which remain poorly understood [35]. A second possibility concerns the formation of protein complexes either via phosphoinositide-binding proteins, or via protein–protein interactions; in the latter regard, the N-terminal non-catalytic region of PI4K IIα contains a large potential helical sequence that might form a coiled-coil, and, intriguingly, several plant PI4K II homologues have multiple ubiquitin-homology domains [36]. Arguably, however, the most probable functions for enhanced PI4K activity in this domain are in vesicular trafficking to and/or from this VCP-rich compartment, as in several other cell membranes [34], and/or in regulating ERAD, as the juxtanuclear localization of cytoplasmic VCP is similar to the reported localization of ERAD substrates following proteasome inhibition or ATP depletion [37,38]. Additional experiments are under way to distinguish between these possibilities.

In summary, these data provide evidence that a large proportion of cellular PI4P synthesis is confined to a discrete intracellular domain, as well as describing potential components of this domain and a basis for its purification.

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