The intracellular localisation and mobility of Type Iγ phosphatidylinositol 4P 5-kinase splice variants

Maria-Luisa Giudici, Koon Lee, Rongxuan Lim, Robin F. Irvine*

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

Received 4 September 2006; revised 14 September 2006; accepted 16 November 2006

Available online 30 November 2006

Edited by Sandro Sonnino

Abstract There are three known splice variants of Type Iγ phosphatidylinositol 4-phosphate 5-kinase (PIPKin Iγ): PIPkins Iγ87, Iγ90, and the most recently cloned (Giudici, M.L., Emson, P.C. and Irvine, R.F. (2004) A novel neuronal-specific splice variant of Type I phosphatidylinositol 4-phosphate 5-kinase isomer gamma. Biochem. J. 379, 489–496) PIPkin IγC (here called PIPkin Iγ93). Here, we have explored the subcellular localisation and mobility of Type I PIPkins in transfected cells by confocal microscopy and fluorescence recovery after photobleaching. The unique behaviour shown by PIPkin Iγ93 is consistent with its suggested distinct function. Moreover, the markedly different localisation and mobility of active versus inactive PIPkin Iγ93 provide insights into the factors that dictate cellular targeting of Type Iγ PIPkins.

Keywords: Phosphatidylinositol 4-phosphate; Phosphatidylinositol 4-phosphate 5-kinase; Fluorescence recovery after photobleaching

1. Introduction

PtdIns(4,5)P₂ is now known to fulfil many intracellular functions (see [1,2] for reviews), and this is reflected in part by the diversity of the family of enzymes principally responsible for PtdIns(4,5)P₂ synthesis [3], the Type I PtdIns4P 5-kinases (Type I PIPkins). The three known mammalian isoforms can exhibit distinct functions (e.g. [4,5] and see [6] for review), and moreover, PIPkin Iγ widens this potential repertoire by existing as three splice variants. Differential splicing of genes is a general way that evolution uses to enhance or modify gene function, by phosphorylation [11,12], and may play an important role in presynaptic vesicle recycling [13,14]. Moreover, a further functional separation of these two splice variants is revealed by the demonstration [15] that PLD2 specifically stimulates integrin-mediated adhesion by a process involving PIPKin Iγ87, but not PIPKinIγ90.

Recently, we extended the known splice variants of PIPkin Iγ by discovering a third spliceoform, which is similar to PIPkin Iγ90, but with an extra 26 amino acids inserted 26 residues from the C-terminus [16]. We originally called this variant PIPkin IγC, but here, to introduce consistency in nomenclature with more extensive recent work on PIPkin Iγ93 (see below), we refer to PIPkin IγC as Iγ93. PIPkin Iγ93 is apparently restricted in its expression to defined neurons in the brain, and we have suggested that it has a unique function in that synthesis a pool of PtdIns(4,5)P₂ which plays a role in the maintenance of neuronal processes [16].

Here, we document a comparative study on the subcellular localisation of all three PIPKin Iγ splice variants. We find that PIPKin Iγ93 has a localisation and mobility entirely different from the other Type I PIPkins, and that its enzymic activity alters its properties.

2. Materials and methods

2.1. Materials

FLAG-constructs were obtained as previously described [16]. GFP-labelled PIPKin Iγ93 was a gift from K.A. Hinchliffe, (Department of Physiology & Pharmacology, University of Manchester, UK).

2.2. Cell culture

COS-7 cells and HEK-293 cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) (Invitrogen, UK) containing 10% foetal bovine serum (FBS) (Invitrogen), 50 U/ml penicillin and 50 U/ml streptomycin at 37 °C, 5% CO₂. Cells were seeded onto glass coverslips (Menzel-Glaser-Germany) pre-coated with poly-α-L-lysine (Sigma, UK) in case of HEK-293 cells.

2.3. Transfection

Transfection of COS-7 cells was done by the calcium-phosphate technique. Cells were incubated overnight with calcium-phosphate/DNA precipitate in DMEM 10%FBS followed by glycerol-shock and then kept in the growth medium.

HEK-293 cells were transfected using FuGene6 Transfection reagent (Roche Molecular Biochemicals, USA).

2.4. Photobleaching

This exactly followed the protocol in Ref. [17].
2.5. Mobile fraction ($M_f$)

The mobile fraction ($M_f$) of a protein is the amount of protein that is free to move within the ROI. In fluorescence recovery after photobleaching (FRAP) experiments, $M_f$ is estimated by the amount of fluorescence recovery in the ROI due to the diffusion of the mobile fluorescent protein from outside the ROI.

The computation of mobile fractions was performed exactly as in Brough et al. [17].

2.6. Diffusion constant

The diffusion constant ($D$, μm$^2$/s) measures the Brownian movement of a fluorescent protein in the ROI over time and was calculated using the Siggia simulation model [18] with software that was a gift from E. Snapp and J. Lippencott-Schwartz see also [17]. $D$ is obtained after comparing experimental and simulated recovery plots. Only experiments with a good fit of experimental and simulated data were accepted to generate the $D$ value.

2.7. Immunofluorescence

This was performed exactly as in Giudici et al. [16].

3. Results and discussion

3.1. Cell transfection

Our initial description of PIPkin I$_{93}$ [16] led us to the conclusion that it is expressed only in the brain, and in only some specific neurons, so for an initial exploration of its localisation we used transfection of cerebellar granule neurons with PIPkin I$_{93}$ constructs. However, we have found these cells unsuitable for exploration of the subcellular localisation of PIPkin I$_{93}$ because of their small size and thin processes. So we turned to COS and HEK-293 cells, which, although subject to the caveat that they do not, as far as we know, normally express PIPkin I$_{93}$, are very much more amenable for investigating subcellular localisation of proteins by confocal microscopy and FRAP. By directly comparing different isoforms and splice variants we can shed indirect but informative light on the properties of Type I PIPkins.

3.2. Localisation of type I PIPkin isofoms in COS cells

In all our experiments described here, we used a short transfection of the cells (4 h maximum), because longer transfections (e.g. 24 h) caused obvious changes in cellular morphology. We believe that by keeping the expression levels to the minimum consistent with detection, we minimise any potential artefacts due to over-expression. When we then transfected GFP- or FLAG-tagged PIPkins I$\alpha$, I$\beta$, I$\gamma$90 and I$\gamma$87 into COS cells, all four of them showed a predominantly plasma membrane localisation (Fig. 1).

We should note that there is some controversy as to the accuracy with which acute transfection of Type I PIPkins reflects their true endogenous distribution. Thus, for example, human PIPkin I$\beta$ (the ortholog of mouse PIPkin I$\alpha$) has been assigned a specific endogenous perinuclear location [4], yet it shows a plasma membrane localisation on transfection ([19,20] and Fig. 1A). A possible explanation is that once the endogenous binding sites for PIPkin I$\beta$ have been saturated, the excess goes by default to the plasma membrane, where its PtdIns4P substrate is located. Indeed, such a plasma membrane localisation caused by substrate binding has been suggested by the work of Kunz et al. [19,20], who, by changing the specificity of a Type I PIPkin (which uses PtdIns4P as a substrate) to that of Type II PIPkin (which uses PtdIns5P), and vice versa, showed that changing substrate specificity of PIPkins can alter their localisation. Their data suggested that it is primarily binding to PtdIns4P in the plasma membrane which dictates the localisations shown in Fig. 1. However, it is important to note that Kunz et al. [20] also showed that this is not the whole story, and some form of protein–protein interaction may also be involved in the plasma membrane localisation of Type I PIPkins.

Whatever the true physiological localisation of these Type I PIPkins, what the data in Fig. 1 show is that in these experiments PIPkin I$_{93}$ (Fig. 1E) is strikingly different from all the four other isoforms or splice variants. It displays a predominantly intracellular localisation, being apparently associated with some kind of structures that are partly vesicular or punctate. We explored its possible co-localisation with cell markers for Golgi, e.r. and mitochondria, and found no convincing co-localisation (not shown). Moreover, although we were able to see some occasional and partial co-localisation with some MVB markers such as EEA-1, a marker of early endosomes, it was not always evident, and certainly did not co-localise with most of the PIPkin I$_{93}$. (Fig. 1). Localisation of Type I PIPkins in COS-7 cells. Confocal images of COS cells transfected with Type I$_{93}$ PIPkin constructs are shown as follows: (A) GFP-PIPkin I$_{93}$; (B) FLAG-PIPkin I$_{93}$; (C) FLAG-PIPkin I$_{93}$ kinase-dead (D316K). Cells were cultured for 2–4 h after transfection, then fixed and stained as described under Section 2.
3.3. FRAP of PIPkins Iγ90 and Iγ87 in HEK-293 cells

In parallel experiments in HEK-293 cells, we noted that the difference between the localisation of PIPkins Iγ90, Iγ87 and Iγ93 was much less obvious (Fig. 2), and in many cells (see Fig. 2C for example) PIPkin Iγ93 appeared similar to Iγ87 and Iγ90 in being mostly close to the plasma membrane. However, more detailed analysis by FRAP revealed that this similarity was largely superficial.

For PIPkins Iγ9 and Iγ87, the majority of protein was mobile, with mobile fractions (Mfs) of 85% and 92%, respectively (Fig. 2A, E and B, F and Table 1). Analysis of the data by measurement of the \( t_{1/2} \) by non-linear regression followed by generation of diffusion coefficients \( (D) \) by the Siggia et al. simulation ([18] and see Section 2), yielded \( D \) values of 0.41 \( \mu m^2 \) s\(^{-1} \) and 0.42 \( \mu m^2 \) s\(^{-1} \) for PIPkin Iγ90 and PIPkin Iγ87, respectively (Table 1). These diffusion coefficients are comparable with the value of \( D = 0.71 \mu m^2 \) s\(^{-1} \) which we obtained in an identical experimental protocol for GFP-GAPI\(^{IP4BP} \) [17], a protein that is probably attached to the plasma membrane by its interaction with PtdIns(4,5)\(_2 \) [21]. However, in discussing those experiments [17] we also raised the possibility that additional interactions with plasma-membrane proteins might account for the lower mobility of GAPI\(^{IP4BP} \) compared with, for example, GAP1\(^{in} \) \( (D = 3.83 \mu m^2 \) s\(^{-1} \)\), when it is transiently attached to the plasma membrane by its interaction with receptor-generated PtdIns(3,4,5)\(_3 \). Indeed, it is interesting that the diffusion coefficients of PIPkins Iγ90 and Iγ87 here are actually closer to the value we obtained \((0.38 \mu m^2 \) s\(^{-1} \)\) for GFP-ICAM, a transplasma membrane protein [17].

We should add that in some earlier experiments we also measured the mobility of GFP-tagged murine PIPkin I\(_x\), and not surprisingly it showed parameters very similar to PIPkins Iγ90 and Iγ87 (D. Brough, M-L.G. and R.F.I., unpublished). Overall, our finding that the mobility of plasma membrane-associated Type I PIPkins is so slow supports the suggestion of Kunz et al. [19,20] that interactions with other membrane proteins also play a role in localising Type I PIPkins to the plasma membrane.

### 3.4. FRAP of PIPkin Iγ93

The mobility of PIPkin Iγ93 was markedly different from the Iγ90 and Iγ87 splice variants. Most of the enzyme was immobile, the mobile fraction being only 36% (Fig. 2C, G and Table 1). This is consistent with PIPkin Iγ93 being confined to vesicular/tubular structures (Fig. 1), such that, although it may diffuse within the vesicle/tubule structure, its recovery within the ROI is governed by the diffusion of the structures themselves through the cytoplasm, which is too slow to be measured over the time course of these experiments. If this is a correct interpretation, then we suggest that these structures are close to the plasma membrane in HEK-293 cells. Whatever the explanation, the difference between PIPkin Iγ90 and the other two splice variants (which are essentially fully mobile) is striking.

We cannot compute a diffusion coefficient for PIPkin Iγ93 in the presence of so much immobile fraction using the Siggia et al. simulation [18], but we were able to gauge an approximate \( t_{1/2} \) for the mobile fraction of PIPkin Iγ93 as about 16 s, similar to that of the almost fully mobile PIPkins Iγ90 and Iγ87, above (Table 1). We assume that this may be a small proportion of PIPkin Iγ93 which is attached to the plasma membrane in a way similar to the other isoforms, but we cannot take this observation further in this context.

![Image of FRAP data from HEK-293 cells](image)

**Fig. 2.** FRAP of Type Iγ PIPkins in HEK-293 cells. Representative FRAP data from HEK-293 cells are shown – quantitative analysis of many cells is given in Table 1. Cells were transfected with the GFP-constructs designated below, and confocal images of representative cells for each are shown in A, B, C and D. For each of these cells, on the right (E, F, G and H, respectively) is shown a typical bleach followed by recovery (see Section 2 for details). Fluorescence values indicated correspond to pre-bleaching (i), immediately after bleaching (ii), and recovery (iii). Constructs are: A/E, GFP- PIPkin Iγ90; B/F, GFP- PIPkin Iγ87; C/G, GFP-PIPkin Iγ93; D/H, GFP-PIPkin Iγ93 kinase-dead.

3.5. Kinase-dead PIPkin Iγ90

We previously employed two kinase-dead constructs of PIPkin Iγ90 as controls (for the effects of kinase-dead PIPkin Iγ93)
in our investigation of cerebellar neurons – two different mutants also controlled for possible non-specific artefacts [16]. When we investigated the localisation of one of these (D316K), it showed a plasma membrane localisation identical to the active PIPkin I90 enzyme (not shown). When we examined it by FRAP in HEK293 cells, the half-life of the kinase-dead enzyme appeared to be different from the active enzyme (Table 1). However, the more rigorous analysis employed to compute D, which takes into account the relative size of the ROI versus the cell and other variables (see [17] for a full discussion of this issue) revealed no significant difference (by T test) between the two enzymes.

This suggests that whatever the contribution of PtdIns4P binding may be to Type I PIPkin localisation [19,20], synthesis of PtdIns(4,5)P2 by the Type I90 PIPkin does not seem to be an important factor in its location in these experiments, or in its interactions as revealed by FRAP.

### 3.6. Kinase-dead PIPkin I93

We also investigated the behaviour of the two different kinase-dead PIPkin I93 mutants that we used in Ref. [16] in both COS and HEK293 cells. In both cell lines the location of D316K and K138A was quite distinct from the active enzyme, appearing to be entirely cytosolic (exemplified by the D316K mutant, shown in Figs. 1F and 2D and H). Moreover, when we investigated the mobility of PIPkin I93 D316K by FRAP the mobile fraction was 92% (Table 1), with a computed D of 3.54 μm²/s⁻¹ (Table 1). The only direct comparison we can make for a cytosolic protein of similar size and dimensions is with our own measurement of GFP-GAP1m when it is cytosolic, which had a diffusion coefficient of 5.56 μm²/s⁻¹ [17], and so within the limits of this technique we can suggest that the kinase-dead PIPkin I93 is probably freely diffusible in the cytosol.

Simplistically, the remarkable difference in localisation and mobility between active and inactive PIPkin I93 has two explanations: Either, (a) the localisation of PIPkin I93, to whatever compartment it is in, depends on its ability to synthesise PtdIns(4,5)P2; or, (b) the endogenous enzyme is truly cytosolic (here faithfully reflected by the kinase-dead construct), and that transfection with an excess of active enzyme has induced an artefactual change in the cells’ physiology, reflected in a changed localisation of the enzyme.

To provide some further insight into this issue we co-transfected live and dead PIPkin I constructs, and the images (Fig. 3) show that this did not alter their subcellular localisation; that is, the kinase-dead PIPkin I93 remains cytosolic even if co-expressed with active PIPkin I93 (Fig. 3C) or active PIPkin I90 (Fig. 3D), and both of the active Type I87, I93 and I90, are unaffected by the co-transfection with kinase-dead PIPkin I93 (Fig. 3A and B, respectively). Thus it seems that it is the actual ability of the enzyme to synthesise PtdIns(4,5)P2 that dictates its localisation, and not a general increase in PtdIns(4,5)P2 in the relevant compartment.

There is an interesting parallel here with the observations of Ling et al. [10,11], who showed a difference in localisation between active and inactive PIPkin I90s, specifically in their localisation to focal adhesions in spreading cells. They suggested that this is probably caused by the lesser degree to which the kinase-dead enzyme is tyrosine phosphorylated, which in turn dictates a decreased association with talin [11,12]. It may be that PIPkin I93 also requires phosphorylation to localise it, and that it needs to be active for this to occur. But if that is so, then for both PIPkin I93 and for PIPkin I90 [10,11], exactly why the enzyme’s ability to synthesise PtdIns(4,5)P2 should alter its efficacy as a substrate for a protein kinase, is a question to which there is no clear answer at present.

### Table 1

| Protein                | Mₜ (%) | t₁/₂ ± S.E.M. (s) | D ± S.E.M. (μm²/s) |
|------------------------|--------|------------------|-------------------|
| PIPkin I90             | 93.16 ± 3.38 (n = 7) | 16.25 ± 2.45 (n = 7) | 0.42 ± 0.13 (n = 10) |
| PIPkin I90             | 84.65 ± 3.94 (n = 8) | 16.03 ± 1.03 (n = 8) | 0.41 ± 0.09 (n = 9) |
| PIPkin I93             | 35.63 ± 3.22 (n = 14) | 15.92 ± 2.89 (n = 14) | –                |
| PIPkin I90 kinase-dead | 77.90 ± 4.50 (n = 10) | 32.74 ± 3.37 (n = 10) | 0.29 ± 0.05 (n = 11) |
| PIPkin I93 kinase-dead | 92.16 ± 1.06 (n = 6) | 4.25 ± 1.42 (n = 6) | 3.54 ± 0.67 (n = 7) |

The table shows the data derived from FRAP experiments on HEK-293 cells transfected with the GFP-tagged constructs shown in the left-hand column. For details see text and Section 2, and for examples of typical cells see Fig. 2.
It is important to note that the mutations that render these PIPkin Iγ constructs catalytically dead target their ATP and Mg2+ binding site [16], and should not affect their interaction with PtdIns4P. So, the difference between localisations of the kinase-dead PIPkin Iγ93 versus the kinase-dead PIPkin Iγ90 (Table 1) provides extra evidence that there is more to the plasma membrane localisation of PIPkin Iγ90 than binding of PtdIns4P. It implies that there must be protein–protein interactions involved, interactions that are presumably prevented by the extra 26 amino acids in PIPkin Iγ93 [16]. This again emphasises the uniqueness of PIPkin Iγ93 amongst the Type I PIPkin family. In conclusion, our data not only highlight the potential complexities of the regulation of the synthesis of different pools of PtdIns(4,5)P2, but more generally illustrate the way in which alternative splicing can generate physiological diversity.

Acknowledgements: We thank Dave Brough for many helpful discussions, and one of the reviewers of the manuscript for useful suggestions. M.-L.G. is supported by a Project Grant from the Wellcome Trust, and R.F.I. by a Programme Grant from the Wellcome Trust and by the Royal Society.

References

[1] Hinchcliffe, K.A., Ciruela, A. and Irvine, R.F. (1998) PIPkins, their substrates and their products: new functions for old enzymes. Biochem. Biophys. Acta 1436, 87–104.
[2] Payrastre, B., Missy, K., Giuriato, S., Bodin, S., Plantavid, M. and Gratacap, M. (2001) Phosphoinositides: key players in cell signalling, in time and space. Cell. Signal. 13, 377–387.
[3] Rameh, L.E., Tolias, K.F., Duckworth, B.C. and Cantley, L.C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature 390, 192–196.
[4] Doughman, R.L., Firestone, A.J., Wojtasik, M.L., Bunce, M.W. and Anderson, R.A. (2003) Membrane ruffling requires coordination between type I alpha phosphatidylinositol phosphate kinase and Rac signaling. J. Biol. Chem. 278, 23036–23045.
[5] Padron, D., Wang, Y.J., Yamamoto, M., Yin, H. and Roth, M.G. (2003) Phosphatidylinositol phosphate 5-kinase IγI recruits AP-2 to the plasma membrane and regulates rates of constitutive endocytosis. J. Cell Biol. 162, 693–701.
[6] Oude Weernink, P.A., Schmidt, M. and Jakobs, K.H. (2004) Regulation and cellular roles of phosphoinositide 5-kinases. Eur. J. Pharmacol. 500, 87–99.
[7] Kopelman, N.M., Lancet, D. and Yanai, I. (2005) Alternative splicing and gene duplication are inversely correlated evolutionary mechanisms. Nat. Genet. 37, 588–589.
[8] Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T. and Oka, Y. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. J. Biol. Chem. 273, 8741–8748.
[9] Di Paolo, G. et al. (2002) Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. Nature 420, 85–89.
[10] Ling, K., Doughman, R.L., Firestone, A.J., Bunce, M.W. and Anderson, R.A. (2002) Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. Nature 420, 89–93.
[11] Ling, K., Doughman, R.L., Iyer, V.V., Firestone, A.J., Bairstow, S.F., Mosher, D.F., Schaller, M.D. and Anderson, R.A. (2003) Tyrosine phosphorylation of type I Gamma phosphatidylinositol phosphate kinase by Src regulates an integrin-talin switch. J. Cell Biol. 163, 1339–1349.
[12] Lee, S.Y., Voronov, S., Letinic, K., Nairn, A.C., Di Paolo, G. and De Camilli, P. (2005) Regulation of the interaction between PIPKI gamma and talin by proline-directed protein kinases. J. Cell Biol. 168, 789–799.
[13] Di Paolo, G. et al. (2004) Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature 431, 415–422.
[14] Morgan, J.R., Di Paolo, G., Werner, H., Shchedrina, V.A., Pypaert, M., Pieribone, V.A. and de Camilli, P. (2004) A role for talin in presynaptic function. J. Cell Biol. 167, 43–50.
[15] Powner, D.J., Payne, R.M., Pettitt, T.R., Giudici, M.L., Irvine, R.F. and Wakelam, M.J.O. (2003) Phospholipase D2 stimulates integrin-mediated adhesion via phosphatidylinositol-4-phosphate 5-kinase Iγb. J. Cell Sci. 118, 2975–2986.
[16] Giudici, M.L., Emson, P.C. and Irvine, R.F. (2004) A novel neuronal-specific splice variant of Type I phosphatidylinositol 4-phosphate 5-kinase isoform gamma. Biochem. J. 379, 489–496.
[17] Brough, D., Bhatti, F. and Irvine, R.F. (2005) Mobility of proteins associated with the plasma membrane by interaction with inositol lipids. J. Cell Sci. 118, 3019–3027.
[18] Siggia, E.D., Lippincott-Schwartz, J. and Bekiranov, S. (2000) Diffusion in inhomogeneous media: theory and simulations applied to whole cell photobleach recovery. Biophys. J. 79, 1761–1770.
[19] Kunz, J., Wilson, M.P., Kisseleva, M., Hurley, J.H., Majerus, P.W. and Anderson, R.A. (2000) The activation loop of phosphatidylinositol phosphate kinases determines signaling specificity. Mol. Cell 5, 1–11.
[20] Kunz, J., Fuellning, A., Kolbe, L. and Anderson, R.A. (2002) Stereo-specific substrate recognition by phosphatidylinositol phosphate kinases is swapped by changing a single amino acid residue. J. Biol. Chem. 277, 5611–5619.
[21] Cozier, G.E., Lockyer, P.J., Reynolds, J.S., Kupzig, S., Bottomley, J.R., Millard, T.H., Banting, G. and Cullen, P.J. (2000) GAP1FβSβ contains a novel group I pleckstrin homology domain that directs constitutive plasma membrane association. J. Biol. Chem. 275, 28261–28268.