Nuclear targeting of the β isoform of Type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its α -helix 7

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Type II phosphatidylinositol phosphate kinases (PIPkins) have recently been found to be primarily phosphatidylinositol 5-phosphate 4-kinases, and their physiological role remains unclear. We have previously shown that a Type II PIPkin [isoform(s) unknown], is localized partly in the nucleus [Divecha, Rhee, Letcher and Irvine (1993) *Biochem. J.* **289**, 617–620], and here we show, by transfection of HeLa cells with green-fluorescent-protein-tagged Type II PIPkins, that this is likely to be the Type II β isoform. Type II β PIPkin has no obvious nuclear localization sequence, and a detailed analysis of the localization of chimaeras

and mutants of the α (cytosolic) and β PIPkins shows that the nuclear localization requires the presence of a 17-amino-acid length of α -helix (α -helix 7) that is specific to the β isoform, and that this helix must be present in its entirety, with a precise orientation. This resembles the nuclear targeting of the HIV protein Vpr, and Type II β PIPkin is apparently therefore the first example of a eukaryotic protein that uses the same mechanism.

Key words: inositides, inositol lipids, NLS, nuclear localization, PIPkin.

INTRODUCTION

The proliferation of potential physiological roles of the inositol lipid PtdIns(4,5) P_2 [1] has, not surprisingly, been accompanied by an increased number of members of the enzyme family responsible for its synthesis. These enzymes are the phosphatidylinositol phosphate kinases (PIPkins) (see [1] for a review). An unexpected twist to this growth of the PIPkin family has been the remarkable demonstration by Rameh et al. [2] that, whereas the Type I PIPkins are indeed PtdIns4 P 5-kinases, as had always been supposed, the Type II PIPkins are 4-kinases, with PtdIns5 P as their preferred substrate. The possibility remains that they may, *in vivo*, be PtdIns3 P 4-kinases (though see [3] for some arguments against this), but most likely either they serve to remove PtdIns5 P , which would imply an as-yet-unknown function for that lipid, or they provide an alternative route of synthesis of PtdIns(4,5) P_2 , presumably representing a form of metabolic compartmentalization to facilitate the many different functions of PtdIns(4,5) P_2 [1,2]. We have shown that the Type II PIPkins are subject to complex regulation [4], including phosphorylation by multiple protein kinases [5], and recently we demonstrated that one isoform, Type II α PIPkin, is subject to a specific control mechanism whereby a serine residue unique to this isoform is phosphorylated by casein kinase II (CK2), and this phosphorylation in turn may cause a translocation of Type II α PIPkin from the cytosol to the plasma membrane [6].

We have previously shown, by Western blotting, that a Type II PIPkin is present in highly purified rat liver nuclei [7], but as our monoclonal antibodies are not isoform-specific, we do not know which isoform is present; the α isoform is predominantly cytosolic in our hands [6], and the γ isoform has been shown to

be mostly in the endoplasmic reticulum [8]. The possible presence of a Type II PIPkin in the nucleus becomes of particular interest when viewed against the background of the increasing complexity of intranuclear lipid signalling [9,10]. For example, there is more than one source of diacylglycerol generated in the nucleus [11,12], and each diacylglycerol pool is regulated differently [12]. Also, as judged by rapid labelling of defined phosphate moieties in the inositol ring, the predominant route of synthesis of PtdIns(4,5) P_2 within rat liver nuclei is by the same route as in whole cells, namely the route one might expect to be catalysed by a Type I PIPkin [2] [PtdIns to PtdIns4 P and then to PtdIns(4,5) P_2] [13]. Thus the presence of a Type II PIPkin, a probable PtdIns5 P 4-kinase, in the same nuclei [7], opens up the possibilities either of a nuclear function for PtdIns5 P , or of two routes of synthesis of PtdIns(4,5) P_2 in the nucleus, possibly for multiple intranuclear functions.

We have found by reverse-transcription PCR that HeLa cells express both the α and β Type II PIPkins (not shown), so to clarify the nature of the Type II PIPkin in nuclei, and to gain some insight into what may take it there [given that none of the cloned Type II PIPkins contain any obvious nuclear localization sequence (NLS)], we have, in a study parallel with the one we conducted on the α isoform [6], transfected HeLa cells with the equivalent green-fluorescent-protein (GFP)-tagged β isoform and investigated its localization.

MATERIALS AND METHODS

N-terminally tagged GFP fusion proteins were produced by ligating Type II α and Type II β PIPkins into the pEGFP-C1 vector (Clontech) as described in [6]. For the wild-type α and β isoforms, C-terminally tagged GFP fusion proteins were also

Abbreviation used: PIPkin, PtdIns5 P 4 kinase (phosphatidylinositol 5-phosphate 4-kinase) (see [1] for a discussion of this abbreviation); NLS, nuclear localization sequence; GFP, green fluorescent protein; mutations are shown using one-letter amino acid codes (e.g. E292P is Glu²⁹² → Pro etc.); CK2, casein kinase II.

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constructed. Site-directed mutagenesis was carried out by the QuickChange site-directed mutagenesis method (Stratagene), and deletions by the seamless method (Stratagene). All constructs were sequenced to confirm the mutations. Plasmids were introduced, by calcium phosphate transfection [14], into HeLa cells grown on coverslips. After 24 h the cells were fixed in 4% (w/v) paraformaldehyde, and mounted in Vectashield (Vector Laboratories, Peterborough, U.K.). The endoplasmic-reticulum staining was carried out, after fixation, with Rhodamine 6G chloride (Molecular Probes). Slides were examined by laser confocal microscopy on a Zeiss confocal microscope.

RESULTS AND DISCUSSION

Transfection of HeLa cells with Type II α or β PIPkins tagged with GFP immediately revealed a distinct subcellular localization, with the α isoform being entirely cytoplasmic (Figure 1a; see also [6]), whereas the β isoform, although detectable in the cytoplasm, was mostly nuclear, with dark patches within the nucleus implying a probable exclusion from nucleoli (Figure 1b).

We should note here that a recent publication by Boronenkov et al. [15] has suggested that both the α and β isoforms of Type II PIPkin are nuclear, apparently in contradiction to our present and previous [6] data on the α isoform. However, all the data presented below are based on short (less than 24 h) transfection, and we have found that longer transfection resulted in significant amounts of the α isoform also being nuclear (results not shown), which we interpret as likely to be an artefact of very high expression levels. Also, the immunocytochemical detection of endogenous proteins by Boronenkov et al. relies on the specificity of an antibody which they stated was not entirely isoform-specific [15]. Moreover, the dimerization of PIPkins [16,17], with its attendant possibility of heterodimerization, leads to the possibility of some α monomers being carried into the nucleus by the nuclear translocation of the β isoform that we show here (discussed further below). In this context we should also note that dimerization will cause the molecular mass of Type II PIPkin to be of the order of 90 kDa, which is too high for it to enter the nucleus by passive diffusion through the nuclear-pore complex even when not tagged with GFP, implying that GFP tagging is not contributing to the contrast between our data and those of Boronenkov et al. [15]. We have tested this implication directly; our monoclonal antibodies to Type II PIPkins [4] have not proved suitable for immunocytochemical localization of endogenous protein, but we have also stably transfected HeLa cells with wild-type (untagged) Type II α PIPkin, and in these cells it was clearly excluded from the nucleus (results not shown). Finally, both α and β isoforms of Type II PIPkin also behaved as in Figure 1 if they were GFP-tagged at the C-terminus (results not shown), suggesting overall that their respective localizations are unlikely to be artefacts of GFP tagging.

To show that this differential localization is also not just a property of this particular cell line, we repeated these transfections in short-term cultured rat hippocampal neurons, and obtained identical distributions of the two Type II PIPkins - α was entirely cytosolic and β largely nuclear (not shown). Co-staining of transfected HeLa cells with Rhodamine 6G chloride, an endoplasmic-reticulum marker (Figures 1c and 1d), revealed that, within the cytoplasm, both isoforms showed no co-localization with the endoplasmic reticulum, suggesting that they are both cytosolic and are thus distinct from the Type II γ PIPkin, which is primarily associated with the endoplasmic reticulum [8].

The Type II β PIPkin has no consensus NLS, so we began making a series of GFP-tagged constructs of chimaeras between

Figure 1 Subcellular localization of GFP-PIPKins

In each panel, examples of cell populations are shown, with each example being representative of 80% of cells transfected with the relevant construct. (a) Wild-type PIPkin Type II α ; (b) Wild-type PIPkin Type II β ; (c) overlay image of (a) and staining for endoplasmic reticulum (in red); (d) overlay image of (b) and staining for endoplasmic reticulum; (e) PIPkin Type II β carrying a deletion between amino acids Cys³⁰⁷ and Asp³²⁵; (f) PIPkin Type II β carrying a deletion between amino acids Gln²⁷³ and Glu³⁰⁶; (g) PIPkin Type II β (E299,303P); (h) PIPkin Type II β (E292P); (i) PIPkin Type II α (N296R,D297A,G298E); (j) PIPkin Type II α (V291M,C293V,N296R,D297A,G298E).

the α and β PIPkins to try to pinpoint what is the NLS in Type II β PIPkin. It soon emerged that the NLS is in the C-terminal half of the β isoform (results not shown), and further chimaeras narrowed down the sequence dictating nuclear localization to the

PIPkin II α	1	MATPGNLGSS	VLASKTKTKK	KHFVAQKVKL	FRASDPLLSV	40	
PIPkin II β	1	MSSNC TSTTAVAVAP	LSASKTKTKK	KHFVCQKVKL	FRASEPILSV	45	
PIPkin II α	41	LMWGVNHSIN	ELSHVQIPVM	LMPDDFKAYS	KIKVDNHLFN	KENMPSHFKF	90
PIPkin II β	46	LMWGVNHTIN	ELSNVPVPVM	LMPDDFKAYS	KIKVDNHLFN	KENLPSRFKF	95
PIPkin II α	91	KEYCPMVFRN	CGKRFGIDVQ	DFQNSLTRSA	PLPND SQARS	GARFHTSYDK	140
PIPkin II β	96	KEYCPMVFRN	LRERFGIDDO	DYQNSVTRSA	PINSD SQGR	GTRFLTYYDR	145
PIPkin II α	141	RYMIKTITSE	DVAEMHNILK	KYHQYIVECH	GITLLPHLLG	MYRLNVDGVE	190
PIPkin II β	146	RFVIKTVSSE	DVAEMHNILK	KYHQFIVECH	GNTLLPQFLG	MYRLTVDGVE	195
PIPkin II α	191	IYVIVTRNVF	SHRLSVYRKY	DLKGSTVARE	ASDKEKAKEL	PTLKDNDFIN	240
PIPkin II β	196	TYMVVTRNVF	SHRLTVHRKY	DLKGSTVARE	ASDKEKAKDL	PTFKDNDFLN	245
PIPkin II α	241	EGQKIYIDDN	SKKVFLERLK	KDVEFLAQLK	LMDYSLLVGI	HDVERAEQEE	290
PIPkin II β	246	EGQKLHVGE	SKKNFLERLK	RDVEFLAQLK	IMDYSLLVGI	HDVDRAEQEE	295
PIPkin II α	291	VECBENDGEE	EGESD...GT	H....PVGT	PPDSPGNTLN	SSPPLAPGEF	332
PIPkin II β	296	MEVEERADEE	ECEND...GV	GGNLLCSYGT	PPDSPGNLLS	FPRFFGPGEF	342
PIPkin II α	333	EPNIDVYGIK	CHENS PRKEV	YFMAIIDILT	HYDAKKKAAH	AAKTVKHGAG	382
PIPkin II β	343	DPSVDVYAMK	SHESSPKKEV	YFMAIIDILT	PYDTKKKAAH	AAKTVKHGAG	393
PIPkin II α	383	AEISTVNPEQ	YSKRFELDFIG	HILT*	408		
PIPkin II β	394	AEISTVNPEQ	YSKRFNEFMS	NILT*	419		

Figure 2 Sequences of the human Type II α and β PIPkins

Sequence identity is shaded. The underlined and boxed regions are explained in the text.

region underlined in the line-up of the two isoforms in Figure 2. Moreover, a mutant Type II β PIPkin with Gln²⁷³–Glu³⁰⁶ deleted was cytosolic (Figure 1f). The isoforms are identical in this region up to Glu²⁸⁴ in the α isoform and Asp²⁸⁹ in the β isoform, which suggests that the boxed region in Figure 2 contains the NLS. This is a particularly interesting place for the possible control of the localization of a Type II PIPkin, as it is the N-terminal part of the region in which the three known isoforms of Type II PIPkins differ most (overall they have 61–78 % similarity; see [1,16]). The recent solving of the three-dimensional structure of the Type II β PIPkin by Hurley's group [16] shows this region of the protein to be a loop, starting (at the N-end) with a 17-amino-acid stretch of α -helix (α -7 in the numbering of Rao et al. [16]), followed by a domain that is unstructured (at least in the crystal). In Type II α PIPkin this loop contains the unique Ser³⁰⁴ residue responsible for translocation to the plasma membrane of that isoform [6], and we should add that our data in that work imply that this loop may undergo specific structural changes to cause the translocation. Thus, overall, this variable loop may represent the major domain for isoform-specific localization and control of Type II PIPkins. The stretch of α -helix that begins this loop is the one that our data point to as possibly determining nuclear localization (boxed in Figure 2).

Deletion of most of the variable loop immediately after α -helix 7 in Type II β PIPkin (Cys³⁰⁷–Asp³²⁵) made no difference to the nuclear localization of that isoform (Figure 1e), which focused our attention on the α -helix 7 itself [16]. Submitting this sequence and its corresponding sequence in the α isoform to analysis for probable secondary structure [18] showed that the equivalent

amino acid residues in the Type II α PIPkin (and the II γ ; results not shown) will not form an α -helix for the entire 17 amino acids, but that, instead, the α -helix will break up after about ten residues [compare Figure 3a (α isoform) with Figure 3b (β isoform)]. We therefore investigated the contribution of the α -helix 7 to the nuclear targeting in two independent ways. First, we introduced amino acid substitutions into this region of the PIPkin β , either two proline residues at positions 299 and 303 (see Figure 2) to disrupt the α -helix structure completely (Figure 3c) or introducing a single proline residue at the beginning of the α -helix [E292P (Glu²⁹² $\rightarrow$ Pro)], which should preserve its overall helical structure (Figure 3d) but change its orientation [16]. The first of these two constructs was entirely cytosolic (Figure 1g), confirming the requirement for the α -helix, and the second resulted in an intermediate distribution; there was clear shift to the cytoplasm, but some nuclear localization was still evident (Figure 1h). The effect of this latter substitution suggests to us that a precise orientation of the α -helix in the Type II β PIPkin is required for nuclear localization.

To establish the contribution of α -helix 7 [16] independently, we introduced an α -helix in stages into the Type II α PIPkin. The 'breakup' of the α -helix occurs about residue Asn²⁹⁶ (Figure 2 and Figure 3a), so we substituted the three residues Asn²⁹⁶–Asp²⁹⁷–Gly²⁹⁸ in Type II α PIPkin with their equivalents, Arg³⁰¹–Ala³⁰²–Glu³⁰³, from the β isoform. Despite the superficial prediction that this should now form an α -helix (Figure 3e), this construct was still entirely cytosolic (Figure 1i). In this construct there are two residues still different in this region from wild-type Type II β PIPkin (Figure 2), namely Val²⁹¹ and Cys²⁹³, which are

The relevant sequences (boxed in Figure 2, and see the text) of some of the constructs used were submitted to analysis for probability of having an α -helical or β -sheet structure by the method of Chou and Fasman [18]. For clarity, only the P_{α} data are shown, as in all examples the P_{β} (propensity to form a β -sheet) data showed the same pattern, but inverted. The central line in each trace shows a P_{α} value of 1.0. **(a)** Wild-type PIPkin Type II α ; **(b)** Wild-type PIPkin Type II β ; **(c)** PIPkin Type II β (E299,303P); **(d)** PIPkin Type II β (E292P); **(e)** PIPkin Type II α (N296R,D297A,G298E); **(f)** PIPkin Type II α (V291M,C293V,N296R,D297A,G298E); **(g)** helical-wheel projection of the β isoform sequence **(b)**, above).

respectively Met²⁹⁶ and Val²⁹⁸ in the β isoform. These are both hydrophobic residues and, simplistically, one would not predict they would have much influence on the structure. However, the

However, there is one precedent for an α -helix being crucial for nuclear localization, and that is in the HIV Vpr protein. Vpr has no ‘classic’ NLS, but apparently interacts with karyopherin α and increases its affinity for basic type (‘classic’) NLSs, and in this way promotes the nuclear translocation of other HIV proteins [23,24]. Recent data on the Vpr protein [25] has shown that disruption of any one of several crucial amino acid residues in its N-terminal α -helix can prevent its nuclear localization. Interestingly, another mutation, not in this α -helix, but close to it (H71R), also prevented nuclear localization (as well as other Vpr functions [25]), and it is an intriguing possibility that this mutation may have changed the orientation of the α -helix in relation to the rest of the protein in a way that might resemble that which we propose for the E292P mutation in Type II β PIPkin (Figures 1h and 3d). Type II β PIPkin may be the first example of a eukaryotic protein using this mechanism of nuclear localization, and other nuclear proteins with no obvious NLS may also use a particular α -helix. In these the central role of an α -helix has not yet been suggested, but this may be difficult to reveal within the structure of a complex protein, and it may be that it is only our fortunate and direct comparison between the very different behaviours of the very similar Type II α and β PIPkins that has enabled us to pinpoint the critical α -helix so precisely in this instance.

Finally, as mentioned in the Introduction, the specific localization of one Type II PIPkin isoform suggests that whatever function this alternative route of $\text{PtdIns}(4,5)\text{P}_2$ synthesis serves, it is likely to apply also within the nucleus. Moreover, if a heterodimerization of Type II PIPkins with consequent translocation of some α or γ monomers to the nucleus (suggested above) can indeed happen, it would make the relative expression levels of the various isoforms of Type II PIPkins in any cell of particular significance, especially when bearing in mind that we have already shown that isomer-specific regulation of the α isoform can take place [6], and that this is mediated by CK2, a protein kinase known to be present partly in the nucleus [26]. Clearly, the regulation of the localization and activity of this intriguing family of proteins is going to be a complex and cell-

specific process, and this is perhaps not surprising, given the overall complexity of inositol-lipid function.

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