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Structural and Functional Diversity of Phosphoinositide 3-Kinases [and Discussion]

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Structural and functional diversity of phosphoinositide 3-kinases

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SUMMARY

Phosphoinositide 3-kinases (PI3-kinases) have been shown to be recruited to cell surface receptor signal complexes whose formation is triggered by growth factors, cytokines and other ligands. PI3-kinases are also involved in protein sorting phenomena. A number of PI3-kinase isotypes have been characterised in several laboratories. Here the relations between the PI3-kinases, PI4-kinases and PI5-kinases and other potential phosphoinositide kinases are analysed. A study of the relation of structure to function for sequence motifs defined through the use of homology searches and protein modelling techniques is described and used to assign the family of phosphoinositide kinases to subgroups.

1. INTRODUCTION

The characterization of receptor signals triggered by growth factors and studies of protein trafficking in yeast have together revealed that 3-phosphorylated inositol lipids generated by a family of lipid kinases may serve as second messengers to trigger disparate functions in cell physiology (reviewed in Kapeller & Cantley 1994).

Fundamental to our current knowledge of the diversity of phosphoinositide function in signal transduction are the classical studies of receptor-linked systems involving G-proteins which showed that the second messengers diacylglycerol (DAG), which activates protein kinase C, and inositol 1, 4, 5-trisphosphate (IP_3), which mediates calcium release, were produced by the action of phospholipase C (PLC) on phosphatidylinositol (4, 5) bisphosphate (PtdIns-(4,5)P_2). A number of the enzymes involved in the biosynthesis of PtdIns(4, 5)P_2, which are PtdIns 4-kinases (PI4-kinases) and PtdIns 4-P 5-kinases (PI5-kinases), have now been characterized from diverse organisms. The realization that phosphoinositides phosphorylated at the D-3 position of the inositol ring could also serve as the source of potential novel second messengers came first from studies of lipid kinase activities associated with transforming proteins of the *src* family (Whitman *et al.* 1988). The new enzymatic activity detected was shown to be a phosphoinositide 3-kinase (PI3-kinase). The involvement of a PI3-kinase activity in receptor signal transduction was found when the enzyme was detected in association with the activated Platelet-Derived Growth Factor (PDGF) receptor. PI3-kinase was subsequently shown to be critically important in PDGF-triggered mitogenesis through the use of cells transfected with mutant receptors which were found to be defective both in their ability to recruit PI3-kinase and to respond to PDGF as a mitogen (Valius & Kazlauskas 1993). It is now clear that the recruitment of PI3-kinase to activated receptor complexes is a common feature of perhaps most cell surface receptors including growth factor and cytokine receptor signalling systems, B- and T-cell receptors and receptors linked to G proteins.

The elucidation of the primary structure of the bovine brain PI3-kinase uncovered structural features which helped clarify the mechanism of recruitment by those receptors which have intrinsic or associated tyrosine kinases (Otsu *et al.* 1991; Hiles *et al.* 1993). A heterodimeric PI3-kinase consisting of a p85 adaptor and p110 catalytic subunits (termed alpha) was shown to be involved. The recruitment of the PI3-kinase is mediated by the src-homology region 2 (SH2) domains of p85 which bind to phosphotyrosine motifs on proteins in the receptor complex (for a review, see Kapeller & Cantley 1994). A second p110 subunit (p110 beta) has been shown to bind p85 (Hu *et al.* 1993). These results showed that the PI3-kinases are encoded by a family of genes and provided clues to the diversity of protein structures found in members of the family.

Subsequent work has revealed other family members

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which can be assigned to new subgroups. The purification and characterization by the Stephens' group (Stephens *et al.* 1994*a*) and the cloning and expression by our group of a G protein-stimulated PI3-kinase (termed p110 gamma) (Stoyanov *et al.* 1995) has shown that a subgroup of PI3-kinases exists which does not use the p85 adaptor subunit. This work has demonstrated that alternative strategies for receptor recruitment and activation of PI3-kinases which have structurally related but distinct catalytic subunits exist.

A second subgroup of PI3-kinases was defined in studies of yeast protein sorting (reviewed in Horazdovsky *et al.* 1995). *Saccharomyces cerevisiae* VPS34 gene encodes a protein required for the sorting of proteins into the yeast vacuole: a compartment similar to the lysosome in higher eukaryotes. The Vps34p is a PI3-kinase with a substrate specificity restricted to phosphatidylinositol (PtdIns) (Schu *et al.* 1993). This was the first clue to the functional diversity in the generation of different 3-phosphoinositides by members of the PI3-kinase family. The partial purification of a bovine PtdIns-specific 3-kinase (Stephens *et al.* 1994*b*) and the cloning and characterization of the activity of a human Vps34p-like enzyme (termed PtdIns 3-kinase) have been described (Volinia *et al.* 1995). This human PtdIns 3-kinase does not use p85, but interacts like Vps34p with a human homologue of the yeast protein Vps15p. This evidence suggests that a protein sorting complex, similar to that found in yeast, having an integral PtdIns 3-kinase, is involved in protein trafficking in the cells of higher eukaryotes.

These studies have shown that the PI3-kinases are a family of proteins which show diversity in their structure, adaptor subunits, function and substrate specificity. In addition there are proteins which have not been shown to be lipid kinases but which contain regions that show sequence similarities to the kinase domain of the PI3-kinases. These include the DNA-dependent protein kinase (Hartley *et al.* 1995), the Ataxia telangiectasia (ATM) gene product (Savitsky *et al.* 1995), other ATM-related proteins (reviewed in Zakian 1995), the yeast TOR proteins (Kunz *et al.* 1993; Hewell *et al.* 1994) and their mammalian homologues FRAP (Brown *et al.* 1994) and RAFT1 (Sabatini *et al.* 1994). Clues to the substrate or structural motifs recognized by the PI3-kinase-like module of these proteins may be gleaned from examination of the primary structure of the active kinases and consequently here we analyse the amino acid sequences of the PI3-kinases using protein sequence analysis, structure prediction and modelling techniques to define subgroups and examine the correlation between structure and function for these enzymes.

2. METHODS

The alignment of amino acid sequences and the generation of dendrograms was performed using the program package MULTALIGN (Barton *et al.* 1987). Secondary structure predictions of the multiple aligned sequences was obtained using the program Zpred (Zvelebil *et al.* 1987). The modelling of three dimensional structure based on crystallographic coordinates was done using the suite of programs available in Quanta™ and InsightII.

3. RESULTS

(*a*) *Conserved regions of amino acid sequence in PI-kinases*

A comparison of the amino acid sequences of the PI-kinases has revealed several motifs which have functional meaning either as conserved motifs in the PI-kinase family, in protein kinases or in other proteins (figure 1*a*). Three homology regions are defined. Homology region 1 encompasses the kinase domain. Homology region 2, previously referred to as the PIK domain, can be found in all PI3-kinases and PI4-kinases. Homology region 3 appears to be specific to PI3-kinases while homology region 4 is restricted to the PI3-kinases of subgroup I. Within the amino terminal region of the subgroup I kinases are motifs that mediate intersubunit interactions with the adaptor and regulatory p85 and a motif involved in the activation of the enzyme by $p21^{ras}$ (ras binding domain, RBD). A further region (ras binding domain homology, RBDH) having homology to the binding site on raf for ras can also be located in PI3-kinases alpha and beta. A ras-GAP homology region which may fold to form a pleckstrin-homology (PH) domain has been found in PI3-kinase gamma.

(*b*) *A dendrogram used to classify PI-kinase domains*

The sequences of the PI3-kinases, PI4-kinases and PI5-kinases together with the sequences of proteins which show a sequence similarity to the PI3-kinases but which have no known enzymatic activity towards phosphoinositides were aligned by using the MULTALIGN program of Barton *et al.* (1987). The sequence information from the large number of protein serine, threonine and tyrosine kinases was used to define the limits of the catalytic domain and to construct a dendrogram of the putative kinase domain, or Homology region I (figure 1*b*).

An examination of the branches of the dendrogram shows that the PI3-kinases which are known to be enzymatically active fall into three subgroups. Subgroup I includes the bovine p110 alpha subunit whose sequence was established first (Hiles *et al.* 1993) together with the closely related human (Volinia *et al.* 1994) and murine p110 subunits (Klippel *et al.* 1994), which may be considered as p110 alpha PI3-kinases. Also found within this subgroup is the human gamma p110 subunit which can be activated by G proteins (Stoyanov *et al.* 1995). This first subgroup also contains two recently characterized PI3-kinases isolated using polymerase chain reaction based techniques from *Drosophila* (L. MacDougall, unpublished results) and human sources (S. Volinia, unpublished results), which now have been cloned and expressed as enzymatically active PI3-kinases.

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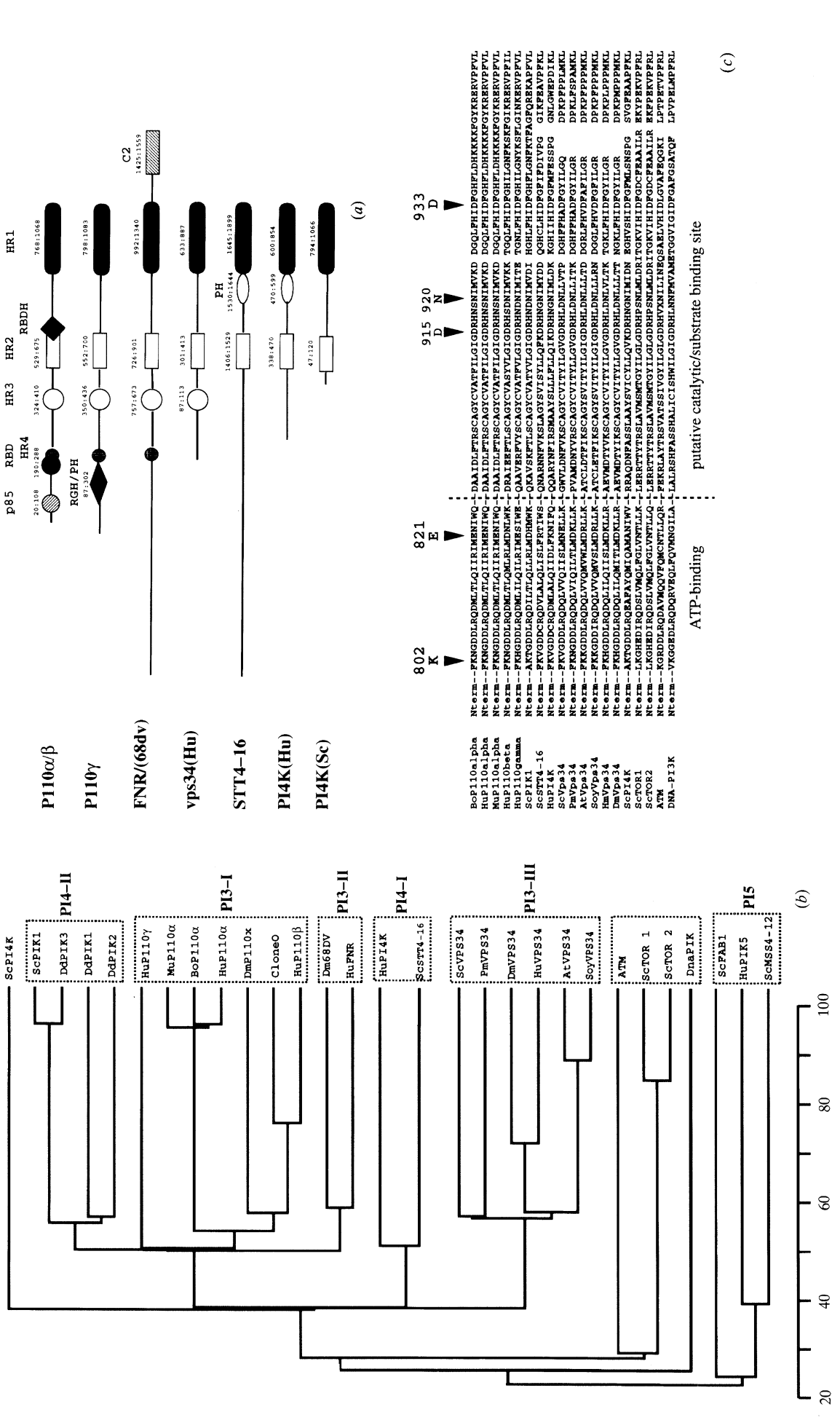

Figure 1. (*a*) The organization of PI-kinase domains. (*b*) A dendrogram of the amino acid sequences of the catalytic domain of PI-Kinases. Dashed boxes illustrate the proposed seperation of groups based on sequence homology and function. (*c*) Local alignment of the ATP-binding and catalytic/substrate binding regions of a representative set of the PI-kinases. References are given in the text.

A second subgroup encompasses a number of PI3-kinases related to the yeast Vps34p PtdIns 3-kinase. Included here are the *Saccharomyces cerevisiae* (Herman *et al.* 1991), *Schizosaccharomyces pombe* (Takegawa *et al.* 1995), human (Volinia *et al.* 1995), *Drosophila* (X. Linasier and L. MacDougall, unpublished data), soybean (Hong & Verma 1994) and *Arabidopsis thaliana* enzymes (Welters *et al.* 1994). The enzymatic activity has been established as specific for the substrate PtdIns for the yeast and human proteins.

The PI4-kinases fall into two subgroups. The first subgroup includes the *Saccharomyces cerevisiae* enzyme, which is encoded by the gene STT4, whose over-expression correlates with increased PtdIns(4)P production (Yoshida *et al.* 1994) and a human PI4-kinase (Wong & Cantley 1994). The second sub-group of PI4-kinases includes the *Saccharomyces cerevisiae* PIK1 125kD gene product whose over-expression results in PtdIns(4)P production (Flanagan *et al.* 1993; Garcia-Bustos *et al.* 1994) and three putative PI4-kinases from *Dictyostelium discoideum* whose enzymatic function has yet to be established (Zhou *et al.* 1995).

The sequences of three PI5-kinases are identified and these fall into a distinct subgroup on the dendrogram. The group includes the human PI5-kinase sequence (Boronenkov & Anderson 1995) and the homologous sequences of the *Saccharomyces cerevisiae* FAB1 gene (Yamamoto & Koshland 1994) and *Saccharomyces cerevisiae* MSS4–12-locus kinase (Yoshida *et al.* 1994*b*).

The analysis of the sequences of the kinase domains of the DNA-dependent protein kinase (Hartley *et al.* 1995), the ATM gene product (Savitsky *et al.* 1995), the *Saccharomyces cerevisiae* TOR1 and TOR2 proteins (Kunz *et al.* 1993) and their mammalian homologues FRAP (Brown *et al.* 1994) and RAFT1 (Sabatini *et al.* 1994) places them in an ill-defined group distinct from the PI3-kinases, PI4-kinases and PI5-kinases.

(*c*) *The function of conserved sequences in the kinase domain of PI-kinases*

The kinase domain of protein kinases was defined by analysis of conserved sequence motifs which are found in 11 linear regions of sequence identified as I to IX (Hanks & Quinn 1991). Specific functional residues from these regions have been located based on extensive biochemical studies and three dimensional information from the X-ray crystallographic structures of protein kinase A (cAPK) (Knighton *et al.* 1991; Zheng *et al.* 1993; Bossemeyer *et al.* 1993), mitogen-activated protein kinase (MAPK) (Zhang *et al.* 1994), cyclin-dependent kinase (CDK2) (DeBondt *et al.* 1993) and the tyrosine kinase domain of the insulin receptor (Hubbard *et al.* 1994). Although the overall sequence homology of the PI3-kinases and the protein kinases is low, there are definite sequence patterns which are retained between these kinases. The best conserved amino acid sequence motifs are implicated in the catalytic activity or co-factor binding function of the kinase. Using the numbering of bovine PI3-kinase alpha, key residues include Lys 802 (cAPK Lys 72) and Glu 821 (cAPK Glu 91), which form a stabilizing ion pair with each other in cAPK. When cAPK binds MgATP, residue Lys 72 comes close to the α and β phosphates of ATP. These residues also form part of the scaffold which make up the core structure. The lysine residue equivalent to Lys 802 of PI3-kinase alpha is invariant in all PI3-kinases and PI4-kinases and is located in a region of high conservation, which is likely to be at or near the substrate binding site. The catalytic loop of cAPK contains invariant Asp (166) and Asn (171) residues which are also retained in the PI3-kinases and PI4-kinases. Asp (166) in cAPK facilitates the rapid release of the phosphopeptide once phosphotransfer has taken place. The equivalent residues in the PI3-kinases and PI4-kinases (Asp 915 and Asn 920 in PI3-kinase alpha) may play a similar role in facilitating the release of phosphoinositides. Residues homologous to those surrounding cAPK Asp 184 are also conserved (see figure 1*c*).

Based on these conserved patterns within the PI3-kinase, PI4-kinase and PI5-kinases and between the PI3-kinase and protein kinases, an alignment has been constructed of p110 alpha and cAPK. To further improve the accuracy of the alignment the secondary structure of p110 alpha, predicted by the program Zpred, and the crystallographically derived secondary structure of cAPK were used as additional constraints in the alignment of the two sequences. The final alignment (45% homology and 11% identity) was used to construct a putative model of the catalytic domain of p110 alpha (Koga *et al.* 1995). Two significantly large insertions with respect to cAMP had to be accommodated within the backbone model of p110 alpha. The first insertion (eight residues) covers part of the potential substrate binding site which in the cAPK X-ray structure contains the PKI-peptide inhibitor. The second insertion (ten residues) appears to replace the N-terminal helix of cAMP which does not seem to be present in the PI3-kinase catalytic subunit. The initial model shows that the conserved residues or patterns form a matching amino acid scaffold with similar hydrogen bonds and electrostatic binding partners. The most prominent difference in the putative catalytic domain of PI3-kinase and the protein kinases is the apparent lack of the otherwise conserved GXGXXG motif that is implicated in ATP binding by protein kinases.

The alignment of the putative catalytic domains of PI3-kinase, PI4-kinase and PI5-kinase shows considerable sequence variation C-terminal to the conserved DFG pattern (see figure 1*c*) that forms part of the substrate binding site. This variation may the result of differences in the substrate specificity of the different PI-kinases.

(*d*) *Conserved sequences in Homology regions 2 (PIK) and 3*

The PIK domain or homology region 2 was first defined in analysis of the yeast PI4-kinase (Flanagan *et al.* 1992). This region is conserved in all the PI3-kinases and PI4-kinases analysed thus far but no function has been correlated with the sequence. Structure prediction studies (Zvelebil *et al.* 1987) suggest that it may consist

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mainly of an α-helical fold. Homology region 3 which has been found only in PI3-kinases has no known function or predictable structure as yet.

(*e*) *The ras binding region of PI3-kinase alpha*

The interaction of p21ras with PI3-kinase alpha, which results in activation of enzyme activity (Rodriguez-Vicinia *et al.* 1994), has been shown by deletion studies of p110 to involve residues 190–288 which we have named the RBD (J. Downward & M. Waterfield, unpublished data). Recently, a 2.2Å X-ray structure of the c-RAF1 ras binding domain complexed with Rap1A was solved (Nassar *et al.* 1995). Local sequence pattern searching was used to obtain possible alignments of the c-RAF1 ras binding domain with the sequence of p110 alpha. The search revealed two regions of homology. The first corresponds to the ras binding domain RBD (residues 190–288) whereas the second identifies another region that could be involved in ras binding (termed here the RBDH region) (figure 1*a*) located partly within the homology region 2 domain.

(*f*) *Subunit interaction domains*

The PI3-kinases which interact with receptors having intrinsic or associated tyrosine kinases are recruited through p85 adaptor subunits which bind particular phosphotyrosine motifs on proteins in the activated receptor complexes. Using deletion studies, the intersubunit binding domain in the p110 alpha and beta catalytic subunits has been located within an amino-terminal including residues 20–108 (Dhand *et al.* 1994*a*). It remains unclear at present if PI3-kinase gamma which is activated by G proteins (Stoyanov *et al.* 1995) has a regulatory subunit. The Vps34p of yeast and the human PtdIns 3-kinase both bind a protein Vps15p which in yeast is a serine–threonine kinase (reviewed in Horazdovsky *et al.* 1995). The regions which mediate the interaction are not yet defined.

The subunit status of the other phosphatidylinositol kinases will be the subject of further investigation and will no doubt reveal several additional mechanisms involved in the control of their activation.

(*g*) *Other interaction domains*

Within the sequences of the PI3-kinase gamma (Stoyanov *et al.* 1995) and a PI4-kinase (Wong & Cantley 1994) lie two regions which may be PH domains. The PH domain of PI3-kinase gamma is most closely related to the PH domain of ras-GAP (Gibson *et al.* 1994). The function of this domain remains unclear although several studies point to a possible role in phosphoinositide or inositolphosphate binding. Clearly such a function could provide both an adaptor and/or regulatory role to the putative PH domains of the PI3-kinase and PI4-kinases where they have been found.

(*h*) *Subgroups of phosphoinositide kinases defined by biological function*

The phosphoinositide kinases can be classified by their function in cell physiology which, in most cases, reflects the function of regions other than the kinase domain as outlined below.

A new class of kinases (PI3-II, see figure 1*a*) having a C2 domain has been isolated using PCR based techniques from both *Drosophila* and human sources (68D, L. MacDougall & M. Waterfield, unpublished results; and fnr, S. Volinia & M. Waterfield, unpublished results).

I. PI3-kinases

PI3-kinases linked to receptors with intrinsic or associated tyrosine kinase activity

p110 alpha (human, mouse and bovine)
p110 beta (human)
p110 (*Drosophila*)

PI3-kinases linked to receptors which activate G protein subunits

p110 gamma (human)

PI3-kinases with C-terminal C2 domains

p210 (human and *Drosophila*)

II. PtdIns 3-kinases involved in intracellular protein sorting or trafficking

S. cerevisiae Vps34p (PtdIns 3-kinase), *S. pombe* Vps34p, human PtdIns 3-K

Drosophila PtdIns 3-kinase, *Drabidopsis* Vps34p and Soybean Vps34p

III. PI4-kinases

Human PI4-kinase, *S. cerevisiae* STT4 and PIK1, *Dicteostileum* PI4-kinases

IV. PI5-kinases

Human PI5-kinase, *S. cerevisiae* FAB1, *S. cerevisiae* MSS4–12

V. Proteins with PI3-kinase-like kinase domains

S. cerevisiae Tor1 and Tor2 and their Human homologues FRAP and RAFT1

DNA-dependent protein kinase (Human)

ATM gene product and the related proteins MEI-41(*S. cerevisiae*), Rad 3 (*S. pombe*), Mec1p (*Drosophila*), Tel1p (*S. cerevisiae*)

References to these proteins can be found in the text.

4. DISCUSSION

The detailed characterization of an ever growing number of phosphoinositide kinases having defined substrate specificities for particular phosphatidylinositols has shown that the enzymes exhibit diverse substrate specificities and distinct subunit structures, and have a number of distinct potentially regulatory modules within their sequences. In addition a significant number of putative phosphoinositide kinases have been discovered based on sequence similarities to

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the phosphatidylinositol kinases with established enzymatic activity. In some cases *in vivo* biological results, such as the altered production of phosphoinositides in yeast overproducer strains, together with significant homology in the kinase and other domains as described above, are sufficient to make a preliminary assignment of the protein to a group of kinases. The search for shared features which define particular groups of phosphatidylinositol kinases as described here is the best that can be done while we wait for three-dimensional structures and more details of substrate recognition features to complement the biochemical studies.

In the future many phosphatidylinositol kinases will be discovered first through the genome project or through other related studies which provide cDNA sequences before any functional data is known. This situation has arisen recently with the discovery of the putative PI3-kinase domain which was reported to lie at the C-terminus of the DNA-dependent protein kinase, the AT gene product and other related genes (reviewed in Zakian 1995). The application of methods for defining the structural features correlated with and diagnostic for the different enzymatic activities of phosphatidylinositol kinases used here, although in their infancy because of the paucity of data, suggest that the DNA-dependent protein kinase and the AT gene product and other related genes would not be expected to have PI3-kinase activity. A search for such activity, using excellent experimental techniques, for the DNA-dependent protein kinase (Hartley *et al.* 1995) failed to find any lipid kinase activity. This suggests that the PI3-kinase like sequences in this group of proteins may be recognizing a different molecule. A similar search for a phosphoinositide substrate for the yeast TOR proteins (Kunz *et al.* 1993) and their mammalian homologues FRAP (Brown *et al.* 1994) and RAFT1 (Sabatini *et al.* 1994) has also been fruitless thus far. The highly specific intrinsic serine kinase activity of PI3-kinase p110 alpha, whose sole substrate in our hands is the p85 adaptor and regulatory subunit (Dhand *et al.* 1994*b*), the observation that the TOR proteins have intrinsic protein/autokinase activity and the specificity of the DNA dependent protein kinase may offer some clues. It is clearly possible that phosphate can be transferred from ATP to serine residues by the PI3-kinase p110 alpha *in vitro* using a kinase domain which can also transfer phosphate to lipids. It is not clear that this function operates *in vivo*, but studies of the structural basis of the phosphate transfer mechanisms employing techniques of sequence analysis used here together with mutagenesis and eventually x-ray crystallography, may resolve the function of the various kinase domains found in lipid kinases and their homologues.

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Discussion

Question. Does Professor Waterfield have any idea what the various unassigned homology regions in the phosphoinositide 3-kinases do?

M. D. Waterfield. The fact that there is conserved sequence across this whole family of phosphoinositide 3-kinases outside the classical kinase domain and the fact that you can only N-terminally truncate p110 by perhaps 100 amino acids suggests that this tightly regulated enzyme has a very complex series of interacting domains that may all be involved in regulation of the kinase domain.

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