Introduction

A large variety of physiological processes, ranging from cytoskeleton dynamics and chromatin remodelling to multiple steps of vesicular trafficking, including regulated secretion, depend upon the presence of members of the lipid family of phosphoinositides [1–4]. In recent years, much effort has been made to identify the protein machinery responsible for their synthesis and degradation [5–8]. What is emerging is a vast collection of gene products with specific localization and regulation, awaiting assembly into a precise map of their cellular distribution. However, we are lacking tools with which to follow specific phosphoinositides and their membrane localization at the cellular level. Three different kinds of phosphoinositide-directed probes could be suitable for this analysis: fluorescent and photoactivatable analogues of inositol phospholipids, phosphoinositide-interacting protein chimaeras, and high-affinity specific antibodies. Here a new protocol for the production and screening of phosphoinositide-specific antibodies is presented, together with a critical evaluation of the results obtained.

Background

The family of inositol phospholipids has recently grown in both number of members identified and importance, and several new functions have been ascribed to many of its members [2,3]. The most representative phosphoinositide is PtdIns(4,5)P₂ [9]. PtdIns(4,5)P₂ was first assumed to play a role only as a precursor of diacylglycerol and Ins(1,4,5)P₃, but in recent years its pleiotropic role in cell physiology has been revealed. PtdIns(4,5)P₂ is a substrate not only for specific phospholipases, but also for phosphoinositide kinases and phosphatases, and thus is at the centre of the metabolic network of inositol phospholipids [9,10]. In addition, PtdIns(4,5)P₂ interacts with a variety of cellular proteins, affecting their localization and activity. PtdIns(4,5)P₂ is a potent mediator of cytoskeletal function and remodelling. In fact, it binds several proteins, including profilin, gelsolin and coflin, which directly regulate actin polymerization. A number of other factors, such as Wiskott–Aldrich syndrome protein (WASP), pleckstrin and myristoylated alanine-rich C-kinase substrate (MARCKS), interact with PtdIns(4,5)P₂, and suggest the presence of a dynamic link between the actin cytoskeleton and the plasma membrane [11–13]. PtdIns(4,5)P₂ also acts as a modulator of the activity of a variety of proteins, such as ADP-ribosylation factor 1 [14] and phospholipase D [15], which have a role in signal transduction and vesicular trafficking [2,3]. PtdIns(4,5)P₂ is also essential for regulated secretion, and it binds several proteins of the molecular machinery responsible for exocytosis and endocytosis, such as synaptotagmin, dynamin and calcium-dependent activator protein for secretion (CAPS) [16,17].

Few attempts have been made to follow the cellular compartmentalization of phosphoinositides directly. Fluorescent analogues of inositol phospholipids were successfully used to prove that phosphoinositides are non-randomly distributed in the membranes of resealed erythrocyte ghost cells [18]. More recently, the ability of the pleckstrin homology domain of phospholipase C-δ to bind PtdIns(4,5)P₂ was exploited to create an intracellular PtdIns(4,5)P₂ sensor via fusion of this pleckstrin homology domain with green fluorescent protein [19,20]. This chimaera has been used to study the distribution of this lipid and its modulation following cell stimulation.

Another possible strategy to follow phosphoinositide dynamics at the cellular level is the preparation of phosphoinositide-specific antibodies. In the past, successful attempts have been made to generate lipid-specific antibodies, including antibodies against polysialogangliosides and several phospholipids [21,22]. In particular, one antibody against the more highly phosphorylated members of the phosphoinositide family has been
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characterized [23]. This antibody was prepared by using a PtdIns(4,5)$_2$ preparation from bovine spinal cord, and also recognizes both the monophosphorylated PtdIns(4,5)$_2$ analogue PtdIns4P and PtdIns(3,4)$_2$P$_2$ [23]. This cross-reactivity is likely to reside in the recognition of an epitope on the lipid backbone common to other members of the phosphoinositide family, and could be due to the heterogeneity of the natural material used in the immunization and screening procedure. No antibodies specific for other PtdInsP, isoforms or for PtdIns(3,4,5)$_3$ are presently available. We have attempted to fill this gap by producing a new series of anti-phosphoinositide antibodies characterized by high specificity.

**Immunization and screening for phosphoinositide-specific antibodies**

We immunized Balb/c mice with liposomes containing 0.5 mg of synthetic dipalmitoyl PtdIns(4,5)$_2$ or PtdIns(3,4,5)$_3$ [24], following an established protocol [25]. Liposomes were prepared with dioleoyl phosphatidylcholine (PtdCho), cholesterol, *Escherichia coli* lipid A and PtdIns(4,5)$_2$ (1:1.5:0.02:0.125, by mol) in PBS, and mice were challenged intramuscularly every 4 weeks with 80 mg of PtdIns(4,5)$_2$ or PtdIns-(3,4,5)$_3$. After spleen-cell fusion, hybridoma supernatants were tested with a new dot-blot assay system. This method, which maintains the advantages of a high-output screening, was developed and used instead of the conventional ELISA system to reduce the number of false-positive clones and to improve the reproducibility of the assay. In this system, 0.5 mg of phosphoinositides or PtdCho dissolved in chloroform/methanol/water (1:1:0.3, by vol.) was spotted on to nitrocellulose in a 12 x 8 array. The lipid spots were allowed to dry and the filters were blocked with 1% (w/v) ovalbumin and 1% (v/v) polyvinylpyrrolidone (PVP) in PBS overnight at 4°C. Membranes were then incubated with hybridoma supernatants, washed and overlaid with horseradish peroxidase-conjugated anti-mouse antibody in PBS containing 3% PVP. After extensive washing in PBS containing 0.05% Tween 20, immunoreactivity was visualized by using a commercial enhanced chemiluminescence protocol.

This dot-blot screening allowed us to select nine positive clones, which were then probed for specificity using the same assay, together with TLC and immunofluorescence protocols. As shown in Figure 1, immunization with PtdIns(4,5)$_2$-containing liposomes produced two hybridomas (10F8 and 2C11) which potently recognized the phosphoinositides, albeit with different sensitivity and specificity. In fact, 10F8 specifically detects PtdIns(4,5)$_2$, whereas 2C11 also recognizes PtdIns4P and PtdIns(3,4,5)$_3$. For both antibodies, no detectable interactions with PtdCho or PtdIns were noted. In addition, 2C11 and 10F8 do not cross-react with genomic DNA, purified histones or protein extracts from isolated nuclei (results not shown).

The ten positive clones were also tested in a TLC-overlay assay performed as previously described [23] with minor modifications. The aluminium-supported silica TLC plates were prepared by running them sequentially in 1.2% potassium oxalate in methanol/water (2:3, v/v) followed by methanol/chloroform (1:1, v/v). After application of the lipid samples, the plates were developed in chloroform/methanol/ NH$_4$OH/water (90:90:20.7, by vol.), air dried and then blocked in PBS containing 1% PVP and 1% ovalbumin. The hybridoma supernatants from selected clones were diluted in the same solution and the plates were processed as described for the dot-blot. As shown in Figure 2, both 10F8 and 2C11 recognized a single band with a migration coefficient corresponding to that of PtdIns(4,5)$_2$, and with no cross-reactivity in this assay system with other phosphoinositides or PtdCho. Migration coefficients of phospholipid standards were determined in parallel by visualization of a section.
Figure 2

Anti-phosphoinositide antibodies specifically recognize PtdIns(4,5)P₂ on a TLC-overlay assay

Portions of 0.5 µg of different phospholipids were spotted on a silica TLC plate and separated as described in the text, before being detected with the phosphoinositide-specific antibodies 2C11 and 10F8. The two antibodies recognized a single spot with a migration coefficient corresponding to that of synthetic dipalmitoyl PtdIns(4,5)P₂.

PtdIns(4,5)P₂-specific antibodies specifically stain interphase nuclei

A further characterization of the anti-PtdIns(4,5)P₂ hybridomas was performed by immunofluorescence on both the HeLa and NIH-3T3 cell lines, following a modified protocol which enhances lipid labelling. After paraformaldehyde fixation (3.7% in PBS) for 10 min followed by two washes in PBS, the coverslips were incubated sequentially in 50 mM NH₄Cl for 15 min and then in PBS containing 2% BSA, 0.25% teleost gelatin, 0.2% glycine and 0.2% Triton X-100 for 1 h. The primary antibody was diluted appropriately in PBS with 1% BSA, 0.25% gelatin and 0.2% Triton X-100, and left to incubate for 1 h. The cells were then washed in PBS containing 0.25% gelatin, and the fluorescent secondary antibody was applied in the same buffer as the primary antibody. After 30 min, coverslips were washed as before and then mounted in Mowiol 4-88. As expected, the ability of the different hybridomas to stain cells in immunofluorescence was strictly correlated with their positivity on the dot-blot and TLC-overlay assays. We found that, in the screening for anti-PtdIns(4,5)P₂ antibodies, only the 10F8 and 2C11 hybridomas presented specific binding above background, and they were therefore selected for further analysis.

As previously stated, one of the most important goals in the study of phosphoinositides is the determination of their cellular compartmentalization and dynamics. In this regard, the availability of a panel of antibodies against the different phosphoinositides will be an invaluable tool. We therefore analysed in detail the distribution of PtdIns(4,5)P₂ in intact cells by using the two monoclonal antibodies 10F8 and 2C11. As shown in Figure 3, both antibodies revealed predominantly non-homogeneous nuclear staining in HeLa and NIH-3T3 cells. The antibodies immunodecorated discrete areas inside the nucleus not in contact with the nuclear membrane; the latter, in general, appeared not to be or very poorly labelled (Figures 3B and 3D). Furthermore, the staining did not overlap with the pattern revealed by the DNA-binding dye Hoechst 33342 [27]. Taken together, these preliminary observations suggest that the nuclear PtdIns(4,5)P₂ is not associated with the majority of the lipid membrane, nor with the DNA. It resides on a still undefined structure present in the nuclear matrix with an overall appearance which resembles the nuclear speckles seen for the nuclear distribution of transcription factors. Preliminary electron microscopic analysis of HeLa cells in the absence of detergent indicates the presence of electron-dense areas in the nuclear matrix which are recognized by the anti-PtdIns(4,5)P₂ antibodies (results not shown). These structures lack any apparent lipid bilayer organization, thus suggesting that PtdIns(4,5)P₂ (and possibly also other phosphoinositides) could be associated with proteins of the nuclear matrix which favour its stabilization outside a typical membrane environment.

The immunoreactivity observed with both phosphoinositide-specific antibodies is totally PtdIns(4,5)P₂-dependent. In fact, the pre-incubation of 2C11 with glycerophospho-D-myo-Inositol(4,5)P₂, which represents the soluble headgroup of PtdIns(4,5)P₂, completely abolished the nuclear staining (results not shown), thus confirming the presence of a significant pool of PtdIns(4,5)P₂ in interphase nuclei.

The presence of phosphoinositides and their biosynthetic machinery in interphase nuclei is well established [1,28-33]. Early evidence for a nuclear phosphoinositide pathway was the identification of a series of phosphoinositol kinase activities both...
in the nuclear membrane and in the nuclear matrix, a discovery soon followed by the localization of phosphoinositide-specific phosphatases and phospholipases [1,31]. Although the physiological significance of an inositol phospholipid pathway in the nucleus is still a matter of debate, it is clear that the nuclear phosphoinositide pool constitutes a significant percentage of total cellular inositol phospholipids. Moreover, their regulation in the nucleus is largely independent of the enzymic machinery present on the plasma membrane. The nucleus therefore constitutes a functionally independent compartment for inositol phospholipid metabolism [11].

The spatial organization of phosphoinositides, their regulatory machinery in the nucleus and the dynamic changes that occur upon gene activation/repression are not known in detail. The association of PtdIns(4,5)P₂ with structures that are insensitive to detergent extraction and are not associated with membrane bilayers strongly suggests the entry of PtdIns(4,5)P₂ in a large protein complex with nuclear localization [28,34]. Recently, the co-localization of PtdIns(4,5)P₂ with phosphatidylinositol phosphate kinase has been demonstrated at nuclear speckles [32], thus supporting the idea that phosphoinositide synthesis and metabolism could be associated with large protein particles in the nuclear matrix. Our new antibodies will be an ideal tool with which to follow the dynamics of these complexes in real time, and will be instrumental for the elucidation of the role of phosphoinositides both inside and outside the cell nucleus.

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Figure 3

Phosphoinositide-specific antibodies reveal discrete nuclear staining

The nuclear staining obtained with the DNA-binding specific dye Hoechst 33342 (A, C) is compared with the labelling with the anti-phosphoinositide-specific antibodies 10F8 (B) and 2C11 (D) in NIH-3T3 cells. Only partial overlapping of the two staining patterns is observed. The nuclear membrane is not stained by either of the antibodies, and only minimal labelling is observed in the cytoplasm and plasma membrane. Scale bar = 2 μm.
Phospholipase C-δ and related molecules

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Introduction

In mammals, the δ isofrom of phospholipase C (PLC-δ) is expressed in a variety of tissues [1]. It has been shown that genes homologous to that encoding mammalian PLC-δ in yeast and plants play important roles in cell growth and/or responses to environmental stress [2,3]. PLC-δ, therefore, could be regarded as an early evolutionary form of PLC. Four subforms of the δ isofrom have been cloned, and termed δ1–δ4 [4–8]. Unlike for the PLC-β and γ isofroms, signalling cascades involving the PLC-δ subforms are still obscure.

Some PLC isofroms, including a newly discovered subform δ4, are present in the nucleus, and are thought to play roles in nuclear signal transduction [7,9]. Little is known about the differential localization and translocation of PLC

Abbreviations used: [Ca2+]i, intracellular Ca2+ concentration; GFP, green fluorescent protein; MDCK cells, Madin–Darby canine kidney cells; PH, pleckstrin homology; PLC, phospholipase C; SHR, spontaneously hypertensive rat.

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