ChemComm

Synthesis and biological evaluation of a PtdIns(3,4,5)P_3 affinity matrix

Gavin F. Painter,[a,b] Jan. W. Thuring,[a,b] Ze-Yi Lim,[a] Andrew B. Holmes,*[a,b] Phillip T. Hawkins[c] and Leonard R. Stephens[c]

[a] *Cambridge Centre for Molecular Recognition, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, UK CB2 1EW. E-mail: abh1@cam.ac.uk; Fax: +44 1223 336362; Tel: +44 1223 336404*

[b] *Melville Laboratory, Department of Chemistry, University of Cambridge, Pembroke Street, Cambridge, UK CB2 3RA. E-mail: abhl@cam.ac.uk; Fax: +44 1223 334866; Tel: +44 1223 334370*

[c] *Babraham Institute, Department of Signalling, Inositide Laboratory, Cambridge, UK CB2 4AT*

Received (in Cambridge, UK) 9th February 2001, Accepted 27th February 2001
First published as an Advance Article on the web 20th March 2001

New PtdIns(3,4,5)P_3 binding proteins have been identified utilising PtdIns(3,4,5)P_3 modified affinity matrix 1 which was synthesised from *myo*-inositol derivative 2, phosphoramidite 9 and an agarose based solid support.

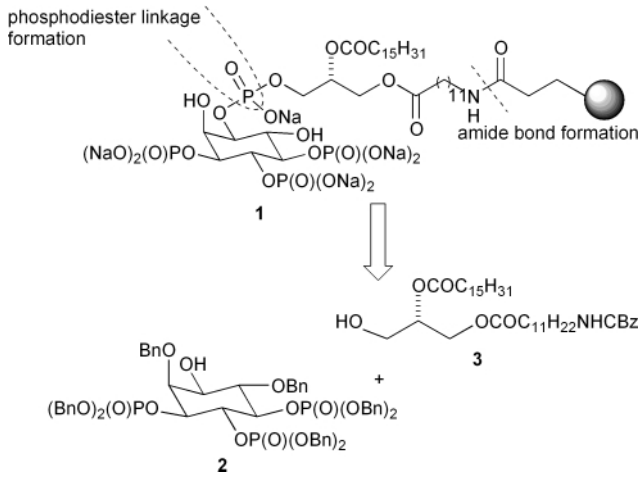
The role of *myo*-inositol phospholipids in cell signalling systems is well established.[1–4] One such signal transduction mechanism involves the *in vivo* production of PtdIns(3,4,5)P_3 *via* phosphorylation of PtdIns(4,5)P_2 mediated by PI3K.[5] Although PtdIns(3,4,5)P_3 binding proteins are known,[6] many of the cellular processes downstream of PI3K activation do not yet have a defined lipid binding protein mapped above them. For this reason we embarked on the preparation and evaluation of an affinity matrix based on PtdIns(3,4,5)P_3.

The phospholipid was attached to an agarose matrix by an amide linkage formed between a carboxylic acid-terminated side chain on the agarose and a 3-(ω-aminoacyl)glycerol derivative on the phospholipid (Scheme 1). The phospholipid **11** was prepared by coupling the alcohols **2**[7] and **3** (from the commercially available (*S*)-(+)-1,2-*O*-isopropylideneglycerol **4**) through a phosphodiester linkage.

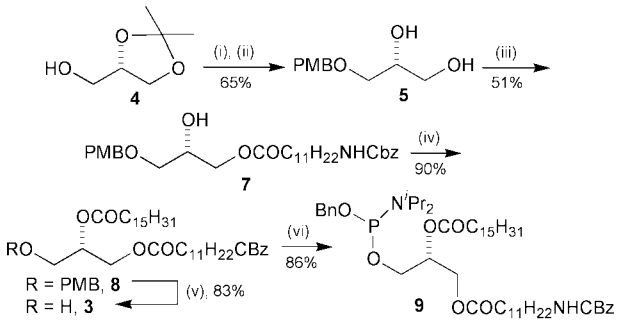
We initially protected the primary alcohol **4** as the *tert*-butyldiphenylsilyl ether, but encountered difficulties in its removal at a later stage of the synthesis. A more efficient process involved 4-methoxybenzylation of the primary alcohol **4** (Scheme 2),[8] followed by acetonide removal to give the PMB-ether **5** (PMB = *p*-methoxybenzyl). Selective esterification of the primary alcohol in **5** with the Cbz-protected (Cbz = benzyloxycarbonyl) ω-amino acid **6**, followed by palmitoylation of the secondary alcohol **7** gave the diester **8**. Oxidative removal (CAN) of the PMB protecting group and phosphitylation of **3** with BnOP(N^iPr_2)$_2$[9] gave the phosphoramidite **9**.†

The lipid side chain, in the form of the phosphoramidite **9**, was then coupled with the enantiomerically pure alcohol (−)-**2** to afford the perbenzylated compound **10** (Scheme 3). Reductive debenzylation was readily effected using H_2 (50 psi) in the presence of Pd-black and $NaHCO_3$ in ^{t}BuOH–H_2O (6:1) as the solvent, to afford the amine **11** in good yield.† This was then coupled with the *N*-hydroxysuccinimide (NHS) activated ester resin, Affi-Gel 10,‡ to afford the PtdIns(3,4,5)P_3 modified matrix **1**. Excess resin (*ca*. 5 equiv.) was required to ensure the complete consumption of the amine which was determined by a negative Kaiser test.§

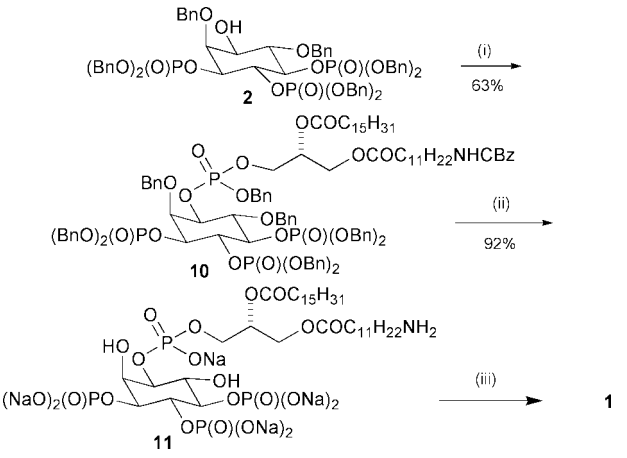
Pilot experiments showed that PKB (25 μM) [a known[6,10] PtdIns(3,4,5)P_3 binding protein] would bind to the matrix **1** and could be completely displaced by 10 μM D,D-PtdIns(3,4,5)P_3¶ but not at all by 10 μM L,L-PtdIns(3,4,5)P_3, thus establishing the potential specificity of the matrix. When applied to a pig neutrophil cytosol a number of proteins have been identified that bind to the resin **1** in a PtdIns(3,4,5)P_3 sensitive manner.



Scheme 1



Scheme 2 *Reagents and conditions*: i, NaH, *p*-MeOC$_6$H$_4$CH$_2$Cl (PMBCl), DMF; ii, PTSA, MeOH; iii, HOOC$_{11}$H$_{22}$NHCBz **6**, DCC, DMAP, CH_2Cl_2; iv, palmitoyl chloride, DMAP, pyridine, CH_2Cl_2; v, CAN, MeCN–H_2O (4:1); vi, BnOP(N^iPr_2)$_2$, 1*H*-tetrazole, CH_2Cl_2.



Scheme 3 *Reagents and conditions*: i, 1*H*-tetrazole, **9**, CH_2Cl_2, then MCPBA; ii, Pd-black, H_2 (50 psi), ^{t}BuOH–H_2O (6:1), $NaHCO_3$; iii, Affi-Gel 10, $NaHCO_3$, H_2O.

DOI: 10.1039/b101320n

Several novel proteins were identified and the full biological results will be disclosed in a separate publication. One of these proteins was subsequently shown to be identical to the recently characterised protein, DAPP1, possessing a Src homology (SH2) domain and a pleckstrin homology (PH) domain. This novel protein had been independently identified from a database search by comparison of the PH domain sequences with known PtdIns(3,4,5)P_3 binding proteins.[11] The fact that the 'functional screen assay' identified several proteins including DAPP1, which is involved in endosomal trafficking or sorting,[12] is noteworthy and exemplifies the strength of the approach. Very recently biotinylated PtdIns(3,4,5)P_3 has been used as an affinity ligand for the purification of recombinant PtdIns(3,4,5)P_3 binding proteins.[13]

In summary we have demonstrated a synthesis of a PtdIns(3,4,5)P_3-modified matrix and demonstrated its use as a tool for the identification of proteins binding to PtdIns(3,4,5)P_3.[14] The flexible nature of the methodology and the biological success of resin **1** warrants further investigation into the preparation and biological evaluation of other D-3 phosphorylated *myo*-inositol phospholipid modified matrices.[15]

We thank the BBSRC, the Cambridge Commonwealth Trust, the CVCP (ORS to Z.-Y. L), Astra-Zeneca and Tan Kar Kee Foundation, Singapore (Z.-Y. L.) for financial support, and the EPSRC for provision of the Swansea Mass Spectrometry Service. We thank Dr Corinne Kaye for helpful advice on the use of agarose supports.

Notes and references

† All new compounds exhibited spectroscopic and analytical data in accord with the assigned structure. *Selected data* (*J* values in Hz) for **9**: $[\alpha]_D^{22}$ + 7.0 (*c* 1.9 in $CHCl_3$); δ_H (250 MHz, $CDCl_3$), 7.38–7.28 (10 H, m, Ph), 5.20–5.10 (1 H, m), 5.10 (2 H, br s, OCH_2Ph), 4.80–4.60 (3 H, m), 4.36 (1 H, m), 4.12 (1 H, m), 3.85–3.55 (4H, m), 3.18 [2 H, dt, (apparent quartet), *J* 6.7, 6.7, CH_2CH_2NH], 2.29 (4 H, two overlapping t, *J* 7.3), 1.64–1.40 (6 H, m), 1.30–1.20 (38 H, m), 1.18 (6 H, d, *J* 6.8, 2 × Me), 1.17 (6 H, d, *J* 6.8, 2 × Me), 0.87 (3 H, t, *J* 6.9, Me); δ_P (101.25 MHz, $CDCl_3$), 149.2, 149.1; *m/z* (FIB) [Found: $(M + Na)^+$ 921.6022. $C_{52}H_{87}N_2O_8PNa$ requires 921.6098]. For **11**: $[\alpha]_D^{22}$ +3.0 (*c* 0.1 in H_2O); ν_{max} (KBr/cm^{-1}) 3403, 2920, 2850, 1742, 1238, 1094; δ_H (250 MHz, D_2O), 5.25 (1 H, br s), 4.45–3.80 (10 H, m), 2.95–2.85 (2 H, m), 2.40–2.25 (4 H, m), 1.65–1.05 (44 H, m), 0.85–0.70 (3 H, m); δ_P (101.25 MHz, D_2O), 5.81, 4.79, 3.55, 0.80; *m/z* (−ve FAB) 1142 [$(M - Na)^-$, 25%], 1119 (50), 1098 (100), 1076 (90).

‡ Affi-Gel 10 was purchased from BioRad.

§ The matrix **1** was constructed by reacting 60 μmol of *N*-hydroxysuccinimide activated resin (4 mL) with 12.2 μmol of the amine **11** in the presence of 122 μmol $NaHCO_3$ at 0 °C overnight.

¶ D,D-PtdIns(3,4,5)P_3 refers to the dipalmitoyl analogue of PtdIns(3,4,5)P_3 containing the 1(D)-*myo*-inositol ring stereochemistry and *sn*-2-diacylglycerol side chain; L,L-PtdIns(3,4,5)P_3 refers to the enantiomer.

1 C. L. Carpenter and L. C. Cantley, *Curr. Opin. Cell Biol.*, 1996, **8**, 153.
2 A. Toker, M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas and L. C. Cantley, *J. Biol. Chem.*, 1994, **269**, 32 358.
3 M. J. Berridge, *Nature*, 1993, **361**, 315.
4 L. R. Stephens, T. R. Jackson and P. T. Hawkins, *Biochem. Biophys. Acta*, 1993, **1179**, 27.
5 C. P. Downes and A. N. Carter, *Cell. Signalling*, 1991, **3**, 501.
6 P. R. Shepherd, D. J. Withers and K. Siddle, *Biochemical J.*, 1998, **333**, 471.
7 G. F. Painter, S. J. A. Grove, I. H. Gilbert, A. B. Holmes, P. R. Raithby, M. L. Hill, P. T. Hawkins and L. R. Stephens, *J. Chem. Soc., Perkin Trans. 1*, 1999, 923.
8 J. Chen, A. A. Profit and G. D. Prestwich, *J. Org. Chem.*, 1996, **61**, 6305.
9 C. E. Dreef, C. J. J. Elie, P. Hoogerhout, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 1988, **29**, 6513.
10 S. R. James, C. P. Downes, R. Gigg, S. J. A. Grove, A. B. Holmes and D. R. Alessi, *Biochem. J.*, 1996, **315**, 709.
11 S. Dowler, R. A. Currie, C. P. Downes and D. R. Alessi, *Biochem. J.*, 1999, **342**, 7.
12 K. Anderson, P. Lipp, M. Bootman, S. H. Ridley, J. Coadwell, L. Rönnstrand, J. Lennartsson, A. B. Holmes, G. F. Painter, J. Thuring, Z.-Y. Lim, H. Erdjument-Bromage, A. Grewal, P. Tempst, L. R. Stephens and P. T. Hawkins, *Curr. Biol.*, 2000, **10**, 1403.
13 D. S. Wang, T. T. Ching, J. St. Pyrek and C. S. Chen, *Anal. Biochem.*, 2000, **208**, 301.
14 P. Hawkins, L. Stephens, S. Ridley, K. Anderson, J. Coadwell, K. Davidson, A. Eguinoa, A. McGregor, M. Manifava, N. Ktistakis, G. Painter, J. Thuring, M. Cooper, Z.-Y. Lim, A. Holmes, S. Dove, R. H. Michell, A. Grewal, H. Erjument-Bromage and P. Tempst, *Nature Cell Biol.*, manuscript in preparation.
15 Z.-Y. Lim, J. W. Thuring, A. B. Holmes, M. Manifava and N. T. Ktistakis, manuscript submitted for publication.