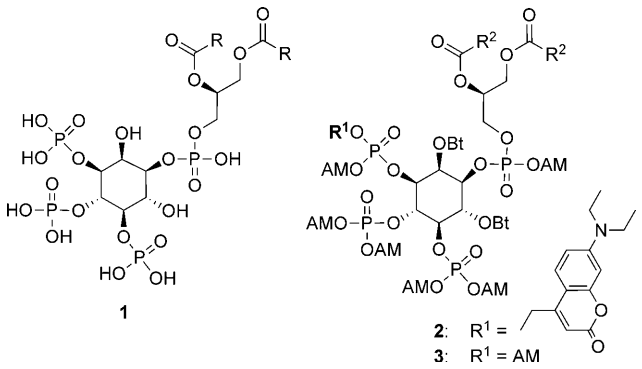
Signal Transduction

DOI: 10.1002/anie.201007796

Photoactivatable and Cell-Membrane-Permeable Phosphatidylinositol 3,4,5-Trisphosphate**

*Matthias Mentel, Vibor Laketa, Devaraj Subramanian, Hartmut Gillandt, and Carsten Schultz**

Phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$ (**1**), Scheme 1)[1] is a second messenger that mediates many intracellular processes including cell proliferation, migration, and survival.[2,3] The lipid is predominantly located in the plasma membrane where it is produced from phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) by several isoforms of PI 3-kinase.[4] This reaction is reversed by the specific lipid phosphatase PTEN.[5] In addition, the $PI(3,4,5)P_3$ phosphatase SHIP removes the 5-*O*-phosphate to produce the metabolite phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$).[6] Owing to the complicated signaling network on the phospholipid level, it is important to be able to manipulate the lipid levels in a specific and rapid way. Silencing of genes by RNA interference experiments or the overexpression of a relevant enzyme is a relatively slow process. An alternative and faster method is the translocation of an intrinsically active enzyme to the membrane, where the phospholipid of interest is located, by the addition of an organic molecule that induces protein dimerization.[7] Another option is the addition of the phospholipid of interest, provided that the lipid will pass the plasma membrane. For phospholipids this requires the use of bioactivatable protecting groups[8] or polyamines.[9] This approach requires the addition of potentially bioactive molecules. In the case of bioactivatable protecting groups, the intracellular enzymatic hydrolysis of acetoxymethyl (AM) esters and butyrates, which is likely to partially occur at membrane interfaces, is slow.[8a,d] This leads to a lipid release that is unphysiologically slow. A solution to this problem is the use of photoactivatable protecting groups (cages).[8c] As the cage prevents biological activity, and ideally hinders rapid metabolism, there is time for the probes to enter cells and for the bioactive protecting groups to be removed. After release of the photosensitive protecting group, immediate activity should be available (Figure 1). If the deprotection (uncaging) could be performed through the lens of a microscope, excellent spatial resolution of the released molecule should be provided.



Scheme 1. Phosphatidylinositol 3,4,5-trisphosphate (**1**) and its membrane-permeable 3-*P*-caged (**2**) and non-caged (**3**) derivatives. R = lipid tail, $R^2 = C_7H_{15}$, AM = acetoxymethyl, Bt = butanoyl.

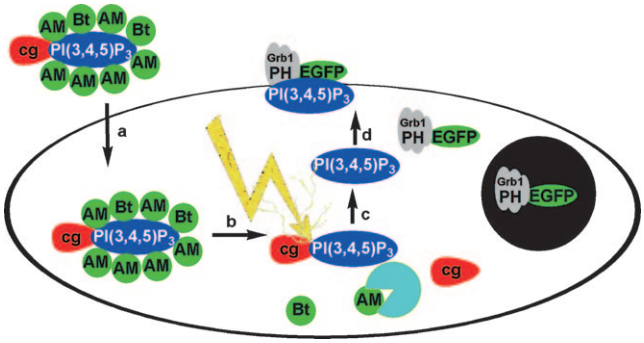


Figure 1. Mechanism of action of **2**: a) Cell entry; b) Enzymatic removal of Bt (butanoyl) and AM (acetoxymethyl) protecting groups produces $cgPI(3,4,5)P_3$; c) Light-induced removal of coumarin protecting group; d) $PI(3,4,5)P_3$ induces translocation of EGFP-Grp1-PH domains to the plasma membrane.

To date, only a few caged lipids have been synthesized[8c,10] and only a single phosphoinositide was among them. Membrane-permeable caged phosphatidylinositol 3-phosphate [cgPI(3)P] was shown to rapidly induce fusion of early endosomes after uncaging.[8c] Herein, we present the synthesis of a membrane-permeable, photoactivatable derivative of $PI(3,4,5)P_3$ ($cgPI(3,4,5)P_3$/AM (**2**), Scheme 1) employing the photoactivatable 7-diethylamino-4-methylenehydroxy coumarin group[11] which worked well for cgPI(3)P. We demonstrate applications in living cells to induce membrane ruffling and PH-domain translocation, two hallmarks of $PI(3,4,5)P_3$

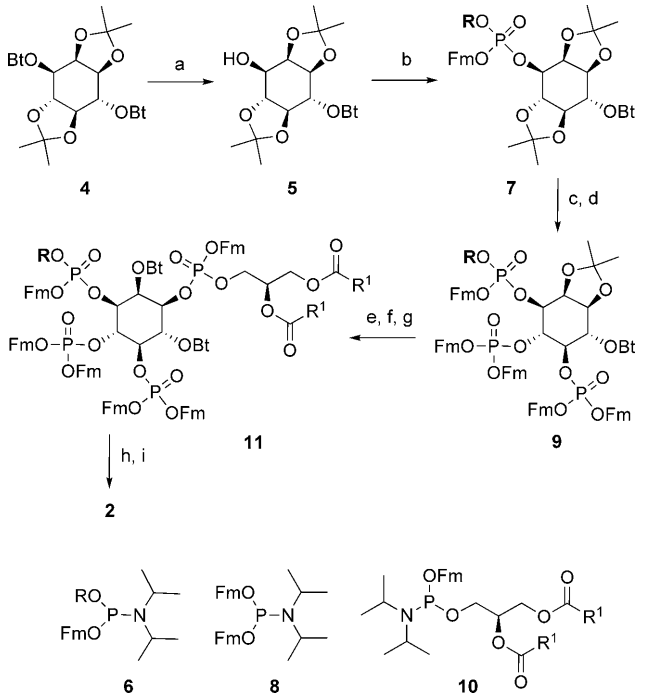
[*] Dr. M. Mentel, Dr. V. Laketa, Dr. D. Subramanian, Dr. C. Schultz
Cell Biology & Biophysics Unit
European Molecular Biology Laboratory
Meyerhofstrasse 1, 69117 Heidelberg (Germany)
Fax: (+49) 6221-387-206
E-mail: schultz@embl.de
Dr. H. Gillandt
Sirius Fine Chemicals (SiChem) GmbH
Fahrenheitstrasse 1, 28359 Bremen (Germany)

[**] We thank the Advanced Light Microscopy Facility at EMBL and Heike Stichnoth for providing cells. Funding was partially provided by SBCancer, the ESF, and the DFG(Schu843/8-1). M.M. is a fellow of the EMBL interdisciplinary postdoc program (EIPOD).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201007796.

signaling, in the presence of the PI 3-kinase inhibitor wortmannin.

As *myo*-inositol is a *meso*-compound with six almost equivalent hydroxy groups, the introduction of two butyrate groups and three differently substituted phosphates is a challenge. Typically, it is difficult to specifically generate single protected or unprotected hydroxy groups, the exception being reactions exploiting electronic or steric differences in the reactivity of *cis*-vicinal hydroxy groups. Our reaction sequence (Scheme 2) started from the dibutyrate of the



Scheme 2. Synthesis of cgPI(3,4,5)P_3/AM (**2**): a) MeOH/Et*i*Pr$_2$N 4:1, 36 °C, 11 h, 91%; b) **6**, 4,5-dicyanoimidazole, CH_2Cl_2, 0 °C to room temperature, 20 min, then AcOOH, −18 °C to room temperature, 1 h, 70%; c) CH_2Cl_2/HCOOH 7:3, room temperature, 3.5 h; d) **8**, 4,5-dicyanoimidazole, CH_2Cl_2/MeCN 4:1, room temperature, 1 h then AcOOH, −18 °C to room temperature, 1.5 h, 48% (2 steps); e) HCOOH/CH_2Cl_2 95:5, 4.5 h; f) Bt(OMe)$_3$, CH_2Cl_2, JandaJel pyridinium trifluoroacetate, room temperature, 23 h then DOWEX 50WX8 H^+, 1 h; g) **10**, 4,5-dicyanoimidazole, CH_2Cl_2/MeCN, room temperature, 30 min then AcOOH, −18 °C to room temperature, 30 min, 46% (3 steps); h) CH_2Cl_2/piperidine, room temperature, 1 h; i) bromomethyl acetate, Et*i*Pr$_2$N, MeCN, room temperature, 10 h, 16% (2 steps). Bt = butanoyl, Fm = 9*H*-fluoren-9-ylmethyl, R = 7-(diethylamino)-2-oxo-2*H*-chromen-4-ylmethyl, $R^1 = C_7H_{15}$.

enantiomerically pure diketal **4**. Selective cleavage of the sterically more accessible 3-*O*-butyrate with the sterically hindered base ethyldiisopropylamine under strict thermal control produced the 3-hydroxy compound **5** in excellent yield. Phosphorylation with the phosphoramidite reagent **6** which contains the photoactivatable coumarin as well a fluorenylmethyl (Fm) group gave the fully protected 3-*O*-phosphate derivative **7** as a pair of diastereomers. In the subsequent steps, the more labile *trans*-ketal group was selectively removed by careful hydrolysis in dichloromethane/formic acid and the resulting diol was rapidly phosphorylated using the di-Fm-phosphoramidite reagent **8**.[12] It became apparent that the coumarin group is an excellent leaving group even under mild conditions, especially when a vicinal hydroxy group was available. Therefore, the next steps needed to be performed in quick succession with only minor purification efforts. After removal of the remaining ketal the diol was subjected to monobutyrylation via a cyclic orthoester intermediate and careful hydrolysis to give the 2,6-di-*O*-butyrate. The 1-*O*-hydroxy group was then treated with the dioctanoylglycerol-bearing P^{III} reagent **10**. The fully protected PI(3,4,5)P_3 derivative **11** was exposed to basic conditions to remove the Fm groups. Alkylation of the resulting phosphate groups with AM bromide gave 25 mg of the hexakis(AM) ester of the caged PI(3,4,5)P_3 (**2**). The final compound was sufficiently stable for analytical characterization. The overall yield of the synthesis was 2.5%, with the biggest losses stemming from coumarin cleavage during the final acetoxymethyl alkylation reaction.

Cellular events regulated by PI(3,4,5)P_3 include, PH domain translocation and the rearrangement of the plasma membrane, often referred to as membrane ruffling.[13] Both effects are easily observable under the microscope by using a GFP-fused PH domain. We preincubated living cells with **2** for at least 1 h to permit enzymatic cleavage of acetoxy methyl esters and butyrates (Figure 1). Illumination with laser light through the microscope objective then released the active phosphoplipid.

As the coumarin cage is a fluorophore ($\lambda_{max} = 380$ nm, $\lambda_{em} = 480$ nm), we were able to demonstrate cell entry into U2OS and HeLa Kyoto cells by fluorescence microscopy and excitation at 405 nm. We observed a distribution into most cellular membranes (Figure S1 in the Supporting Information). Next, we expressed an EGFP-fusion of the Grp-1 PH-domain in U2OS cells, a known reporter of plasma membrane PI(3,4,5)P_3 levels.[14] Uncaging of cgPI(3,4,5)P_3 (5 or 10 μM extracellularly, 1 μM was ineffective; Supporting Information, Figure S2) by a short pulse of 375 nm laser light through the microscope objective resulted in translocation of the cytosolic PH domain to the plasma membrane within one minute (Figure 2), about the same time required by platelet-derived growth factor (PDGF) to induce membrane ruffling and PH-domain translocation (Supporting Information, Figure S3). In comparison to the use of non-caged compound **3** or histone-PI(3,4,5)P_3 conjugates, cgPI(3,4,5)P_3 (**2**) was the only successful compound (Supporting information, Figure S4). Interestingly, we observed that the elevated PI(3,4,5)P_3 levels in this and the following experiments were accompanied with a strong translocation of the labeled PH domain from the nucleus to the cytosol (Figure 2, and Figures S3, S5, S7 in the Supporting information).

Alternatively to a pulsed activation, and causing less stress to the cells, we illuminated HeLa cells every 15 s with low-intensity light at 405 nm (laser power 2%) through the objective. Again, Grp1-PH-GFP translocated to the plasma membrane within a few minutes (Supporting information, Figure S5). To quantify the Grp1-PH-GFP translocation to the plasma membrane after uncaging and to compare it with PDGF stimulation we used total internal reflection microscopy (TIRF). TIRF allows the exclusive visualization of the

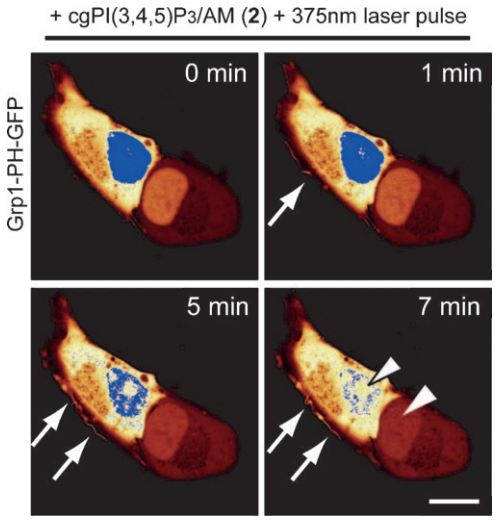


Figure 2. Fluorescence microscopic images of living U2OS cells treated with wortmannin. Treatment with **2** (10 μM, 1 h) had no effect. Illumination with a 5 s laser flash induced membrane ruffling and PH-domain translocation to the membrane folds (arrows) within 1 min. In addition, nuclear PH domains translocate to the cytoplasm (triangles). The blue color indicates saturating fluorescence intensity. Scale bar 10 μm.

plasma membrane owing to the strong focality of the evanescent field (100 nm depth) and is hence the most quantitative way of imaging PH-domain translocation and membrane ruffling (Figure 3, Figure S6). cgPI(3,4,5)P_3 uncaging and PDGF stimulation by flash photolysis induced a similar onset of PH-domain translocation (Figure 3) demonstrating that the release of PI(3,4,5)P_3 mimics part of growth-factor signaling, as was previously shown for the uncaged PI(3,4,5)P_3 derivative **3**.[8b] Pre-incubation with cgPI(3,4,5)P_3

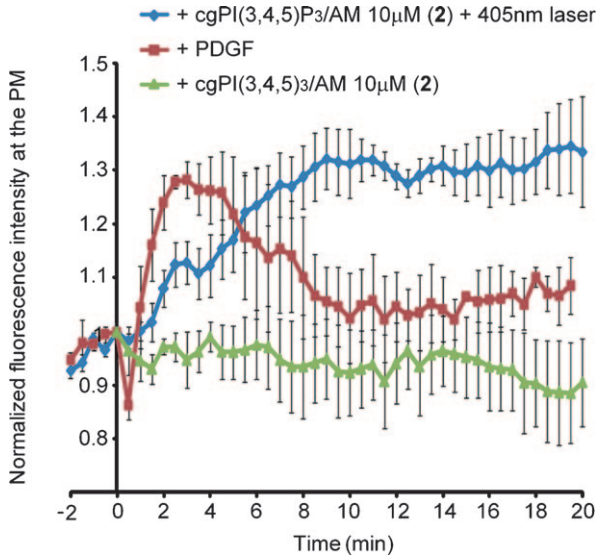


Figure 3. Time course of PH-domain translocation in HeLa cells without uncaging of **2** (green), after uncaging of **2** (blue), and after addition of PDGF (red). Intensity values are normalized to time zero before uncaging. Error bars represent the standard deviation measured from three different cells.

did not have any effect on PH-domain location in the absence of photolysis (Figure 3, green line). Cellular effects were observable even after several hours of pre-incubation and subsequent uncaging suggesting that metabolism of the caged compound **2** is fairly slow (Supporting information, Figure S7).

One of the advantages of caged compounds is a release with spatial resolution. HeLa cells showed a significant increase in membrane ruffling when illuminated globally. When one part of a single cell was illuminated within the field of view, the illuminated region showed more membrane ruffling than the other parts of the cell, demonstrating that local elevation of signaling lipids is possible (Figure 4). The laser power required for successful uncaging was surprisingly low. Higher doses of light or illumination after higher doses of **2** (100 μM) often led to cell death.

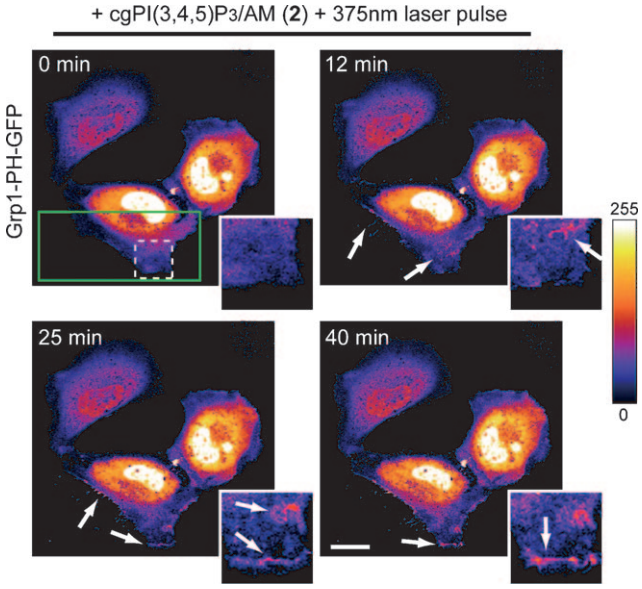


Figure 4. Local photoactivation of **2** (10 μM extracellularly) produces a local response in U2OS cells. A locally limited (rectangular green box) 5 s laser pulse initiated membrane-ruffling as detected by Grp1 GFP-PH-domain translocation (red coloration) predominantly in the illuminated region. Other cells were unaffected. Scale bar 10 μm.

In conclusion, we have demonstrated the first successful synthesis of a photoactivatable, membrane-permeable PI-(3,4,5)P_3 derivative. The choice of the photochemically favorable coumarin cage required especially mild and optimized chemical procedures for the protecting group and phosphorylation chemistry. Nevertheless, the overall yield was respectable. The immediacy and strength of the biological response is clearly superior to that of the membrane-permeable, but uncaged derivative.[8b] In the future, more photoactivatable phosphoinositide derivatives of other lipids including sphingosines and cholesterol need to be developed to help understand the distinct interplay of lipid-induced activities. In addition, photoremovable groups sensitive to longer wavelength are urgently needed to allow the use of several caged compounds within the same experiment and to

reduce potential photodamage in long-term experiments. We are looking forward to a large number of biological experiments where cgPI(3,4,5)P_3/AM (**2**) is used to manipulate intracellular PI(3,4,5)P_3 levels in a temporally and spatially resolved fashion.

Received: December 10, 2010
Published online: March 14, 2011

Keywords: caged compounds · phospholipids · kinases · signal transduction

[1] a) A. E. Traynor-Kaplan, A. L. Harris, B. L. Thompson, P. Taylor, L. A. Sklar, *Nature* **1988**, *334*, 353–356; b) A. E. Traynor-Kaplan, B. L. Thompson, A. L. Harris, P. Taylor, G. M. Omann, L. A. Sklar, *J. Biol. Chem.* **1989**, *264*, 15668–15673.
[2] D. A. Cantrell, *J. Cell Sci.* **2001**, *114*, 1439–1445.
[3] L. C. Cantley, *Science* **2002**, *296*, 1655–1657.
[4] K. R. Auger, L. A. Serunian, S. P. Soltoff, P. Libby, L. C. Cantley, *Cell* **1989**, *57*, 167–175.
[5] P. A. Steck, M. A. Pershouse, S. A. Jasser, W. K. A. Yung, H. Lin, A. H. Ligon, L. A. Langford, M. L. Baumgard, T. Hattier, T. Davis, C. Frye, R. Hu, B. Swedlund, D. H. F. Teng, S. V. Tavtigian, *Nat. Genet.* **1997**, *15*, 356–362.
[6] J. E. Damen, L. Liu, P. Rosten, R. K. Humphries, A. Jefferson, P. W. Majerus, G. Krystal, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 1689–1693.
[7] a) D. J. Austin, G. R. Crabtree, S. L. Schreiber, *Curr. Biol.* **1994**, *1*, 131–136; b) G. R. Crabtree, S. L. Schreiber, *Trends Biochem. Sci.* **1996**, *21*, 418–422; c) J. D. Klemm, S. L. Schreiber, G. R. Crabtree, *Annu. Rev. Immunol.* **1998**, *16*, 569–592; d) T. Inoue, W. D. Heo, J. S. Grimley, T. J. Wandless, T. Meyer, *Nat. Methods* **2005**, *2*, 415–418; e) P. Varnai, B. Thyagarajan, T. Rohacs, T. Balla, *J. Cell Biol.* **2006**, *175*, 377–382.
[8] a) C. Schultz, *Bioorg. Med. Chem.* **2003**, *11*, 885–898; b) V. Laketa, S. Zarbakhsh, E. Morbier, D. Subramanian, C. Dinkel, J. Brumbaugh, P. Zimmermann, R. Pepperkok, C. Schultz, *Chem. Biol.* **2009**, *16*, 1190–1196; c) D. Subramanian, V. Laketa, R. Müller, C. Tischer, S. Zarbakhsh, R. Pepperkok, C. Schultz, *Nat. Chem. Biol.* **2010**, *6*, 324–326; d) M. Bartsch, M. Zorn-Kruppa, N. Kuhl, H.-G. Genieser, F. Schwede, B. Jastorff, *Biol. Chem.* **2003**, *384*, 1321–1326.
[9] S. Ozaki, D. B. DeWald, J. C. Shope, J. Chen, G. D. Prestwich, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 11286–11291.
[10] C. Dinkel, O. Wichmann, C. Schultz, *Tetrahedron Lett.* **2003**, *44*, 1153–1155.
[11] V. Hagen, J. Bendig, S. Frings, T. Eckardt, S. Helm, D. Reuter, U. B. Kaupp, *Angew. Chem.* **2001**, *113*, 1077–1080; *Angew. Chem. Int. Ed.* **2001**, *40*, 1045–1048.
[12] a) Y. Watanabe, T. Nakamura, H. Mitsumoto, *Tetrahedron Lett.* **1997**, *38*, 7407–7410; b) L. Bialy, H. Waldmann, *Chem. Eur. J.* **2004**, *10*, 2759–2780.
[13] G. Di Paolo, P. De Camilli, *Nature* **2006**, *443*, 651–657.
[14] T. Balla, T. Bondeva, P. Várnai, *Trends Pharmacol. Sci.* **2000**, *21*, 238–241.

