Subcellular Localization and Structural Function of Endogenous Phosphorylated Phosphatidylinositol 4-Kinase (PI4K92)*

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Anti-phosphopeptide antibodies were raised against phosphatidylinositol 4-kinase (PI4K92) phosphorylation sites (Suer, S., Sickmann, A., Meyer, H. E., Herberg, F. W., and Heilmeyer, L. M. Jr. (2001) Eur. J. Biochem. 268, 2099–2106). Characterization proved three of them (anti-pSer-294, anti-pSer-496, and anti-pThr-504 antibody) to be highly specific, recognizing solely PI4K92 phosphorylated at these sites, respectively. Indirect immunofluorescence reveals that PI4K92 phosphorylated on Ser-294 localizes exclusively at the Golgi. The enzyme phosphorylated on Ser-496 and Thr-504 is detected in nuclear speckles. Phosphorylation of Ser-294 on PI4K92 increases the lipid kinase activity and thus serves better in maintaining Golgi function and morphology (compare Haussler, A., Storz, P., Martens, S., Link, G., Toker, A., and Pfizenmaier, K. (2005) Nat. Cell Biol. 7, 880–886). Microinjection of anti-pSer-496, but not of anti-pSer-294 or anti-pThr-504 antibody, into the cytoplasm or into the nucleus of HS68 cells leads to development of hotspots, probably representing aggregated PI4K92, and in later stages, cells become apoptotic and finally die. The association of phosphorylated PI4K92 with nuclear speckles is dynamic and follows the morphological alteration of speckles upon inhibition of mRNA transcription with α-amanitin. Overexpressed PI4K92 phosphorylated on Ser-294 is not transported to the nucleus, and that phosphorylated on Ser-496 is found in the nucleus and mislocalized at the Golgi complex. We conclude that nuclear phosphatidylinositol 4-phosphate, and consequently, synthesis of polyphosphoinositides are required for a correct nuclear function.

In the past 20 years, evidence has been accumulated for the presence of intranuclear polyphosphoinositides that form the components of a phosphoinositide-phospholipase C cycle (for review, see Ref. 1), which is independent from that at the plasma and other cytoplasmic membranes (2, 3). In a recent study, the pleckstrin homology domain of phospholipase C θ1 has been used as a probe to show that phosphatidylinositol (4,5)-bispahosphate (PtdIns(4,5)P2) is present intranuclear and is not detectable in the nuclear membrane (4). Alternatively, a monoclonal antibody used for indirect immunofluorescence staining procedures reveals PtdIns(4,5)P2 in distinct subnuclear domains, identified as “nuclear speckles” (Ref. 5 and 6, and see also Refs. 7 and 8). Most probably, these latter polyphosphoinositides are not present in membranes but are associated with proteins (2, 3, 9). In principal, it explains why they are not extractable with Triton X-100, which, however, completely removes the nuclear double membrane (9).

Several distinct functions are well known for polyphosphoinositides in the cytoplasm in addition to their role as precursor for the generation of second messengers, inositol (1,4,5)-trisphosphate and diacylglycerol. In the middle of the 1990s, it was revealed that PtdIns(4,5)P2 itself may serve as a regulator or effector molecule on its own right (for review, see ref. 10). In many cell types, stimulation with growth factors or hormones results in marked changes in cellular morphology. This alteration is thought to be due to a rearrangement of actin via influencing actin-binding proteins such as profilin, coflin, α-actinin, vinculin, talin, and ezrin by binding to polyphosphoinositides (11).

In addition, PtdIns(4,5)P2 plays an important role in membrane trafficking. It is suggested to regulate exocytotic fusion of synaptic (glutamate release) and dense core vesicles (dopamine release) with the plasma membrane (12, 13). Besides PtdIns(4,5)P2, the phosphatidylinositol 4-phosphate (PtdIns(4)P) and the generating enzyme, phosphatidylinositol 4 kinase (PI4K), seem to be directly involved in secretion processes and have an essential role in regulating clathrin-dependent endocytosis, phagocytosis, pinocytosis, and endosome motility (14–17). However, little is known about the alternative function of PtdIns(4,5)P2 in the nucleus other than that it is a precursor in the phosphoinositide cycle.

In nuclei stripped of their envelope with detergent, PI4K and phosphatidylinositol 4-phosphate 5-kinase as well as diacylglycerol kinase activities are reported to be associated with the nuclear matrix using biochemical approaches (18). Phospholipase C β as well as protein kinase C appear to co-localize on the nuclear matrix as shown by immunoelectron microscopy (3, 19). More precisely, phosphatidylinositol 4-phosphate kinase isoforms, type I, i.e., the phosphatidylinositol 4-phosphate 5-kinase (PI4K), seem to be present in the nuclear matrix. In nuclei this may be due to a rearrangement of actin via influencing actin-binding proteins such as profilin, coflin, α-actinin, vinculin, talin, and ezrin by binding to polyphosphoinositides (11).

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Molecular details of several PI4K isoforms are known today. These are PI4K230, PI4K92 (also known as PI4Kβ), and PI4K55 (for review, see Ref. 20). By blocking nuclear export with leptomycin B (LMB), two of these isoforms have been shown to accumulate in the nucleus, PI4K230 (21) and PI4K92 (17). Within the nucleus, the native PI4K230 seems to be localized in the nucleolus, whereas no specific nuclear localization has been reported for PI4K92. In addition, PI4K92 has been shown to exert an important function in maintaining the structural integrity of the Golgi complex (16).
In the intact cell, we have shown that PI4K92 can be phosphorylated on eight sites; however, nothing is known on the function of these phosphorylation sites so far (22). Here we will demonstrate that specifically phosphorylated forms of PI4K92 are associated with speckles in the nucleus and that an alternatively phosphorylated form is associated with the Golgi apparatus in the cytoplasm. Moreover, injection of anti-PI4K92 antibodies directed against a specific phosphorylation site into the nucleus leads to cell death, underlying the important function of the PI4K92 for cell survival.

**MATERIALS AND METHODS**

**Reagents**—Anti-HA monoclonal antibody (HA.11), monoclonal antibody against nuclear envelope pore complex protein and polyclonal anti-giantin and anti-actin antibody were from Covance Inc. Anti-PI4Kβ antibody was from Upstate Biotechnology, and anti-Sc-35 antibody was from Sigma. The monoclonal anti-golgin97 antibody and the fluorophore-conjugated secondary antibodies were from Molecular Probes. Peroxidase-conjugated polyclonal anti-rabbit and monoclonal anti-mouse antibodies were from Amersham Biosciences. Peroxidase-conjugated Protein A was from Pierce. Monoclonal antibodies against PtdIns(4,5)P2 and phosphatidylinositol trisphosphate (PtdIns(3,4,5)P3) were from Echelon. Cell culture material was from Sigma. Standard molecular biological tools were from Stratagene if not designated otherwise. All other chemicals were of high performance liquid chromatography or analytical grade.

**Phosphorylation State and Site-specific Antibody Production and Purification**—Individually designed polyclonal anti-phosphopeptide anti-sera and synthetic peptides corresponding to sequences of human PI4K92 were obtained from NEOSYSTEM (Strasbourg, France). The following phosphopeptides (and cognate non-phosphopeptides) were used: H₂⁻KRTAS(Po₃)NPKVY-OH (pSer-294); H⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻/>. 

**Total Cell Lysate and Western Blot Analysis**—Cultured cells were scraped into denaturing lysis buffer (50 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.25% (w/v) SDS, 0.25% (w/v) deoxycholate, 1% (v/v) Nonidet P-40, 1 mM EDTA) containing protease inhibitors (final concentrations: 10 μg/ml aproteinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and protein phosphatase inhibitors (final concentrations: 20 mM NaF, 20 mM β-glycerophosphate, 1 mM Na2VO4, 1 mM okadaic acid, and 1 μM microcystin) or non-denaturating lysis buffer (instead of 0.25% SDS, 0.25% deoxycholate, and 1% Nonidet P-40, it contained 1% Triton X-100). Cell homogenate was passed through a 26-gauge needle few times and centrifuged at 20,000 × g for 20 min at 4 °C. Supernatant was assayed for protein concentration by the Lowry method. Collected supernatant of non-denaturing cell lysate was employed for immunoprecipitation followed by lipid kinase activity assays.

Aliquots of denaturating lysates containing equal amounts of protein were subjected to SDS-PAGE analysis and Western blotting. The polyvinylidene difluoride membrane was blocked using 5% milk powder in Tris-buffered saline (TBS)(50 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for at least 1 h at room temperature. As primary antibody, anti-PI4Kβ antibody was used at 0.1 μg/ml in 1% milk powder in TBS, or affinity-purified anti-phosphopeptide antibodies were used at 1–10 μg/ml. The membrane was rinsed two times and then washed three times in TBS for 10 min and subsequently incubated with secondary antibody using peroxidase-conjugated donkey anti-rabbit antibody (Amersham Biosciences) at the dilution of 0.005 μg/ml in 1% milk powder in TBS for 1 h. After washing, the binding of antibodies was detected with enhanced chemiluminescence (ECL Advance; Amersham Biosciences) and visualized using x-ray film (Amersham Biosciences). For antibody blocking experiments, affinity-purified anti-phosphopeptide antibodies were preincubated for 2 h at room temperature with 20 μg/ml respective phospho- or dephosphopeptide.

**Immunoadsorption**—Unspecific binding sites of Sepharose beads conjugated with anti-rabbit antibody (Sigma) were preblocked with 3% (v/v) bovine serum albumin in phosphate-buffered saline (PBS) for 2 h at 4 °C. 5 μg of anti-PI4Kβ or affinity-purified anti-phosphopeptide antibodies were added to equal protein concentration containing aliquots of cell lysate and incubated for 2 h at 4 °C with continuous rotation. The antigen-antibody complex was collected by adding 40 μl of preblocked anti-rabbit antibody-conjugated Sepharose beads. Control experiments included mock immunoprecipitation in the absence of antibody and the use of anti-giantin IgG. After 2 h of incubation, beads were washed with 2 × 0.5 ml of lysis buffer, 2 × 0.5 ml of lysis buffer additionally containing 400 mM NaCl, and 2 × 0.5 ml of PBS. Samples were subjected to Western blot analyses, or alternatively, immunoadsorbsent on Sepharose beads were analyzed for PI4K92 activity.

**PI4K92 Activity Assay and Product Analysis**—Incorporation of radioactivity from [γ-32P]ATP into extractable organic solvent material was measured as described previously (24). Produced phospholipids were extracted according to Ref. 25. The chloroform phase was transferred to scintillation vials or spotted onto TLC plates NH2 (Merck, Darmstadt, Germany). Plates were developed in 1-propyl acetate/2-propanol/ethanol/6% aqueous ammonia (3:9:3:9, v/v) (26) and visualized by PhosphorImager (Amersham Biosciences).
In Vivo Metabolic Labeling—Cos7 cells were grown on 10-cm diameter dishes. One day before phosphate deprivation, cells were transfected with 8 μg of PI4K92-WT plasmid DNA at a density of 80% confluence. At the start of phosphate deprivation, cells were washed three times with sodium phosphate- and sodium pyruvate-free Dulbecco’s modified Eagle’s medium containing 0.5% dialyzed fetal calf serum and than incubated for 10 min. After phosphate deprivation, cells were metabolically labeled with [32P]orthophosphate (ICN) at a concentration of 1 mCi/ml in phosphate-free medium containing 0.5% dialyzed serum for 4 h. After labeling, cells were incubated with 100 μM calyculin A in phosphate-free medium for 10 min followed by washing three times with ice-cold PBS containing calyculin A. Cells were lysed in 0.5 ml of denaturing lysis buffer. Metabolically labeled cell lysate was used for immunoabsorption followed by Western blotting to detect the amount of PI4K92, the same membrane was probed with anti-PI4K antibody (0.1 μg/ml) followed by detection with peroxidase-conjugated secondary antibody.

Cell Treatments, Transfection, and Immunofluorescence—Human skin fibroblasts, cell line HS68, and Cos7 cells, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 10 μg/ml gentamycin. For immunofluorescence microscopy, HS68 cells or transfected Cos7 cells were grown on chamber slides (Nunc) and were fixed with 4% (v/v) formaldehyde in PBS (Sigma) for 20 min at room temperature. After five washes with PBS (5 min each), fixed cells were incubated in blocking and permeabilization solution (5% serum from the host animal of the secondary antibody and 0.2% Triton X-100 in PBS) for 30 min. This solution was also used for diluting the primary and secondary antibodies. Cells were sequentially incubated with primary and then fluorophore-labeled secondary antibodies for 1 h, respectively, at a concentration suggested by the manufacturer. Cells were washed five times with PBS containing 0.2% Triton X-100 after antibody incubations. Following the last washing step, the cells were postfixed with 4% (v/v) formaldehyde in PBS and then mounted with ProLong Antifade Kit (Molecular Probes). For inhibition of transcrip-
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FIGURE 2. Localization of the Ser-294 phosphorylated PI4K92 at Golgi complex. Cells were double-stained as described under “Materials and Methods” with anti-pSer-294 antibody (a) and with anti-Golgin97 antibodies (b and e). The merged images are shown in panels c and f. The anti-pSer-294 antibody was preincubated with 20 μg/ml corresponding phosphopeptide (d). Bars, 10 μm.

RESULTS

Anti-phosphopeptide Antibodies Specifically Recognize Phosphorylated Forms of PI4K92—Anti-phosphopeptide sequence-specific antibodies were generated by immunizing rabbits with synthetic phosphopeptides covalently coupled to ovalbumin. Each peptide was built of four amino acids up- and downstream of the phosphoamino acid according to the described specific amino acid sequences of PI4K92 (22). It was certified that these peptides are unique among known phosphopeptide sequences and that they are a typical feature of PI4K92 according to BLASTP 2.2.5. data base search (29).

In the ELISA tests, affinity-purified antibodies (see “Materials and Methods”), anti-pSer-294 (Fig. 1A, panel I), anti-pSer-496 (Fig. 1A, panel II), and anti-pThr-504 antibodies (Fig. 1A, panel III), react exclusively with the relevant phosphopeptides but not with the dephosphopeptides. Preimmune sera show no immunoreactivity. A commercially available anti-PI4Kβ antibody directed against residues 410–537 of human PI4K92, including the phosphorylation sites pSer-496 and pThr-504, does not yield a signal neither with the appropriate phosphopeptide nor with dephosphopeptide (not shown).

In whole cell extracts prepared from HS68 human fibroblast cells (Fig. 1B), these three above mentioned affinity-purified anti-phosphopeptide antibodies selectively detect a 92-kDa protein corresponding to PI4K92, which was certified with the commercially available antibody against human PI4K92 (anti-PI4Kβ, later termed control antibody). In Cos7 cells containing recombinant PI4K92 (Fig. 1B), only the anti-pSer-294 and the anti-pSer-496 antibody detect the 92-kDa protein, whereas no reactivity was observed with the anti-pThr-504 antibody. Immunolabeling of the 92-kDa PI4K is inhibited by preincubation of antibodies with the appropriate phosphopeptide but is unaffected by preincubation with dephosphopeptide (Fig. 1B).

Transfected Cos7 cells overexpressing wild type PI4K92 were labeled with [32P]orthophosphate (see “Materials and Methods”). A radioactively labeled 92-kDa protein is immunoprecipitated with anti-pSer-294 and anti-pSer-496 antibodies as well as with the control anti-PI4K92 antibody (Fig. 1C, X-ray). The protein bands detected by autoradiography were identified as PI4K92 by Western blotting with the control antibody (Fig. 1C, W8). In agreement with the Western blotting experiments reported in Fig. 1B, no reactivity was shown with the anti-pThr-504 antibody.

Anti-phosphopeptide-antibodies immunoabsorb lipid kinase activity (Fig. 1D) from HS68 cell lysate prepared under non-denaturating conditions. More of the lipid kinase activity is precipitated with
anti-pSer-294 antibody than with the control anti-PI4K92 antibody (defined as 100%). The anti-pSer-496 antibody precipitates approximately an equal amount of kinase activity, whereas anti-pThr-504 antibody yields much less. Based on parallel Western blotting experiments, it can be shown that the majority of the PI4K92 protein was obtained with the control anti-PI4K92 antibody (Fig. 1E), whereas anti-pSer-294 as well as anti-pSer-496 and anti-pThr-504 antibodies immunoprecipitate much less (Fig. 1E).

The assay for PI4K activity contains 0.4% Triton X-100 to block phosphatidylinositol 3-kinase activity. In agreement, all of the immunoprecipitated PI4K92 enzymes produce exclusively PtdIns(4)P. In a TLC system that allows separation of D3 and D4 phosphorylated derivatives, only the D4 phosphorylated species is detected (not shown). Taken together, these data demonstrate that these affinity-purified anti-phosphopeptide antibodies selectively and specifically recognize their and just their adequate phosphorylation site of native PI4K92; thus, they are suitable for localization studies by immunocytochemistry.

Native Specifically Phosphorylated PI4K92 Reveals Cytoplasmic and Nuclear Localization—Immunofluorescent staining with affinity-purified anti-pSer-294 antibody shows the presence of Ser-294 phosphorylated PI4K92 at the Golgi structure (Fig. 2a). The identified subcellular structure is verified by merging the images obtained with anti-pSer-294 antibody with that of the Golgi marker, anti-Golgin97 antibody (Fig. 2c). When the anti-pSer-294 antibody was preincubated with an excess of phosphopeptide encompassing the Ser-294 phosphorylation site, this Golgi-associated staining disappeared completely (Fig. 2d), whereas incubation of the marker antibody retained the Golgi staining pattern (Fig. 2e). Thus, the phosphopeptide itself does not prevent the Golgi staining. No change in staining pattern was observed by preincubation with dephosphopeptide (not shown).

The anti-pSer-496 antibody recognizes a speckled nuclear component (Fig. 3a). Indeed, as revealed in the merged image, anti-pSer-496 antibody co-localizes with a marker antibody directed against a splicing

![FIGURE 3. Localization of the Ser-496 phosphorylated PI4K92 at nuclear speckles.](http://example.com/figure3.png)
factor, Sc-35, which is a subcomponent of nuclear speckles (Fig. 3c). Thus, in the nuclear speckles, the Ser-496 phosphorylated enzyme is present. Preincubation with the corresponding phosphopeptide eliminates this staining completely (Fig. 3d), whereas again, staining with the marker antibody is uninfluenced (Fig. 3e). Merging these two micrographs does not change the anti-Sc-35 antibody staining pattern (Fig. 3f). No co-localization was observed with the marker antibody for nuclear envelope and a Golgi protein as shown in the micrographs (Fig. 3, i and l). Essentially, an identical behavior is seen with the anti-pThr-504 antibody (Fig. 4). Again, the image obtained with the anti-pThr-504 antibody merged with that obtained with the anti-Sc-35 antibody verifies the speckle localization. In addition, preincubation with the corresponding phosphopeptide prevents speckles staining with the anti-phosphopeptide antibody but not with the anti-Sc-35 antibody. No co-localization is observed with either nuclear envelope or the Golgi apparatus.

Inhibition of RNA polymerase II with α-amanitin causes reorganization of the nuclear staining pattern into fewer and larger structures, as revealed by staining with anti-pSer-496 as well as with anti-pThr-504 antibodies (Fig. 5, d and e). An identical rearrangement of staining pattern is seen with the anti-Sc-35 antibody (Fig. 5f).

In Cos7 cells, inhibition of nuclear export with LMB leads to accumulation of the overexpressed enzyme in the nucleus as revealed by staining with anti-HA antibody (Fig. 6, a and e). The anti-pSer-294 antibody detects the phosphorylated form of the enzyme at the Golgi apparatus but no enzyme in the nucleus (Fig. 6b). The merged picture shows that the Ser-294 phosphorylated enzyme stays on the Golgi, whereas the non-phosphorylated form at this site is transported into the nucleus (Fig. 6c). The opposite is seen with the anti-pSer-496 antibody. Nuclear staining is observed with both the anti-phosphopeptide as well with the anti-HA antibody. In addition, both antibodies stain the Golgi apparatus, which never has been observed with the endogenous enzyme (Fig. 6).
Microinjection of Anti-pSer-496 Antibody into HS68 Cell Results in Cell Death—Microinjected anti-pSer-496 antibody into the cytoplasm of HS68 cells is not translocated to the nuclear fraction but causes cell death with a half-time of $\sim 18$ h (Fig. 7, a and b). Neither the cytoplasmic actin filaments nor the DNA structure change due to the presence of these antibodies in the cytoplasm within a period of 7 h.

The anti-pSer-496 antibody was microinjected into the nucleus, where it shows initially an even distribution (Fig. 7c). After $\sim 7$ h, anti-pSer-496 antibody targets 6–20 hotspots in the nucleus, independent of the nucleoli, in G1 phase (Fig. 7h) and a limited number of hotspots in G2 phase cells (not shown). Nuclear hotspots become significant as cells begin to die. After 24 h following the microinjection, a large aggregate of the PI4K92 evolves, and cells undergo apoptosis (Fig. 7k). Finally, the nuclear staining collapses; however, DNA does not condense. In addition (Fig. 7i), super bundling of actin occurs due to the stress induced by the antibody present in the nucleus (Fig. 7m).

Nuclear injection increases the speed in which cells die. Heat-inactivated or control antibody anti-pSer-294 has no effect (not shown).

In another series of experiments, anti-pSer-496 antibodies were microinjected into the nucleus of HS68 cells, and after $\sim 10$ h, the cells were stained with anti-PtdIns(4,5)P2 and anti-PtdIns(3,4,5)P3 antibodies. Fig. 8 shows that the microinjected cell (Fig. 8a) develops hotspots and that this cell exhibits a much lower amount of nuclear PtdIns(4,5)P2 (Fig. 8b) than the non-injected control cells (compare Fig. 8, a and b), indicating that the anti-pSer-496 antibody inhibits the production of PtdIns(4,5)P2 due to inhibition of PtdIns(4)P synthesis. However, the level of PtdIns(3,4,5)P3 in cytoplasmic membranes including Golgi does not show any change. Thus, the nuclear decrease is not reflected in the level of PtdIns(4,5)P2 in cytoplasmic membranes (compare injected and non-injected cells in Fig. 8b). The level of PtdIns(3,4,5)P3 is much lower in the nucleus; therefore, the decrease of this phospholipid following microinjection of anti-pSer-496 antibody is hardly to be detectable (not shown).
Microinjection of anti-PtdIns(4,5)P₂ (Fig. 8d) leads to aggregates of PI4K92 as seen previously (compare Fig. 7k). Indeed, the cells die as well. Heat-inactivated antibody has no effect (not shown). It allows the conclusion that microinjected anti-pSer-496 antibody blocks the production of PtdIns(4)P, and as a consequence, the production of the higher phosphorylated phosphatidylinositol derivatives, which, however, seem to be absolutely required for a correct nuclear and cellular function.

DISCUSSION

Phosphorylated PI4K92 was detected in mammalian cells previously without defining its phosphorylation status (30). Eight phosphorylation sites were determined on the His₆-tagged human PI4K92 isoform purified in its native form from Sf9 insect cells by mass spectrometry (22). However, no function could be assigned to any of these eight phosphorylation sites until today. Therefore, in this study, polyclonal antibodies were generated against each phosphorylation site taking the sequence information described by Ref. 22. As proposed in Refs. 31 and 32, we synthesized nonameric peptides comprising four amino acids up- and downstream from the phosphorylated amino acid in question. Some of these peptides overlap; however, no cross-reactivity was ever observed in ELISA tests (not shown). Five antibodies showed strong cross-reactivity with proteins other than PI4K92 (not shown), which were, therefore, eliminated from further studies. In contrast, three affinity-purified anti-phosphopeptide antibodies (anti-pSer-294, anti-pSer-496, and
anti-pThr-504) selectively recognize a single 92-kDa band, like the control anti-PI4K92 antibody. The phosphorylation site and state specificity was verified by ELISA tests, by immunocompetition, by immunoabsorption of PI4K92 activity, and by immunoabsorption of in vivo \(^{32}\)P-labeled phosphoprotein.

The enzyme activity assays after immunoabsorption with anti-pSer-294, anti-pSer-496, and anti-pThr-504 antibody clearly show that these antibodies do not inhibit the PI4K92 activity. Moreover, these experiments have revealed that phosphorylation of PI4K92 definitely occurs on a rather low level. There is much less PI4K92 immunoadsorbed by the anti-phosphopeptide antibodies than by the control anti-PI4K92 antibody. Since the PI4K92 phosphorylated on Ser-294 and on Ser-496 expresses an even higher activity than that precipitated by the control antibody, the phosphorylated enzyme must have a higher specific activity than the average immunoadsorbed enzyme by the control antibody representing a mixture of all non- and phosphorylated enzyme forms.

The evaluation for Thr-504 phosphorylation is undefined due to the low amount of precipitated protein. Nothing can be said in relation to the phosphorylation state of the five sites not characterized here, which might influence the specific activity as well. Thus, it can be concluded that three of the eight anti-phosphopeptide antibodies (anti-pSer-294, anti-pSer-496, and anti-pThr-504 antibody) strictly recognize only the specifically phosphorylated forms of PI4K92.

Phosphorylated PI4K92 on Ser-294 Is Associated to the Golgi Complex—With these three anti-phosphopeptide antibodies, a correlation between the phosphorylation of specific sites and the location of the native enzyme could be shown in the cell. First, the Ser-294 phosphorylated species is exclusively observed at the Golgi. Indeed, it has been shown that PI4K92 is required to maintain a native Golgi structure and function (16, 17, 30). For this function, a highly active and Ser-294 phosphorylated form seems to be necessary. It is interesting to note that this phosphorylated form expresses a higher specific activity than the average enzyme including all different phosphorylated forms, which might also be important for the integrity of the Golgi structure, especially since a dead mutant of PI4K92 does not maintain a correct Golgi structure (16).

The Ser-294 phosphorylated enzyme has never been detected in the nucleus. Even when the enzyme was overexpressed and nuclear export was blocked with LMB, the Ser-294 phosphorylated enzyme remains only on the Golgi. One of several interpretations could be that the Ser-294 phosphorylated form cannot be imported into the nucleus or is actively exported from the nucleus. Of course, it cannot be excluded that other phosphorylation sites on the enzyme or multiple phosphorylated forms are responsible for this effect.

Phosphorylated PI4K92 on Ser-496 and on Thr-504 Localize to the Nuclear Speckles—A surprisingly clear location of the native enzyme has been found in the nucleus with the two anti-phosphopeptide antibodies, anti-pSer-496 and anti-pThr-504, namely on a specific nuclear component, the speckles. A faint nuclear staining of PI4K92 without defining a specific location within the nucleus has been described previously (17). Nuclear import and export signals within the sequence of the enzyme have been proposed (17, 20). Indeed, inhibition of nuclear export with LMB leads to massive accumulation of the enzyme in the nucleus (17). Again, no specific location within the nucleus has been detected in these experiments. Even in this non-physiological condition, only the Ser-496 phosphorylated form is found in the nucleus but not the Ser-294 phosphorylated form, as shown here. Nothing can be said concerning the Thr-504 phosphorylated form because this antibody does not recognize the recombinant protein in Cos7 cells. However, the overexpressed enzyme, phosphorylated on Ser-496, is also found at the Golgi, where the native Ser-496 phosphorylated form of the enzyme has never been found. Thus, the overexpressed enzyme is mislocalized there. However, it shows that Ser-496 can be phosphorylated in the cytoplasm. The protein kinase responsible for the phosphorylation of Ser-496 is protein kinase A (33), which is present, as is well known, in the cytoplasm. It is not known, however, whether the native enzyme can be phosphorylated in the nucleus as well (33). Unfortunately, microinjection of the catalytic subunit of protein kinase A or its corresponding inhibitor, protein kinase A inhibitor, did not provide clues to this question (experiments not shown). Even less is known about the location of Thr-504 phosphorylation; no protein kinase could be assigned for catalyzing this reaction (22).

The presence of PI4K92 on speckles is consistent with the finding that a large set of enzymes of the phosphatidylinositol cycle as well as their products are detected there (i.e. PIPKs type Ia and type IIa isoforms as...
well their product, PtdIns(4,5)P₂ (1, 5); phosphatidylinositol 3-kinase Class 2α and 2β (34, 35); SHIP-2 (36, 37)). Thus, the whole phosphatidylinositol cycle can occur on speckles. It is interesting to note that the phosphatidylinositol cycle occurs in the nucleus on a non-membranous structure, the speckles, whereas in the cytoplasm, it is restricted to membranes.

The nuclear speckle morphology correlates with transcriptional activity; speckles are small and more diffuse when pre-mRNA synthesis occurs and fewer and larger when transcription is inhibited (38–40). It was demonstrated that the PIPks and their product, PtdIns(4,5)P₂, reorganize identically with speckles, both spatially and temporally, suggesting direct interaction of PIPks with speckle component(s) (5). An identical reorganization pattern was observed in the case of Ser-496 and Thr-504 phosphorylated PI4K92 (Fig. 7).

Microinjection of anti-pSer-496 antibody leads to cell death either when injected into the cytoplasm or much faster when microinjected into the nucleus. This seems to be due to the lowering the PtdIns(4)P level, and as a consequence, of PtdIns(4,5)P₂ on the speckles (Fig. 8).

Apparently, the enzyme is phosphorylated on Ser-496 in the cytoplasm and transported to the nucleus. This transport might be inhibited by the presence of these antibodies recognizing and binding to the phosphorylated Ser-496 site in the cytoplasm. By this way, the enzyme might be trapped in the cytoplasm, and therefore, the level in the nucleus is decreased critically. Indeed, phosphorylation of the enzyme on Ser-496 in the cytoplasm has been shown by overexpressing it and mislocalizing it on the Golgi (compare Fig. 7).

Microinjection into the nucleus causes a disorganization, seen as hotspots, or in later stages, as collapsed nuclear structure. Hotspots can be seen as early as 4 h and their product, PtdIns(4,5)P₂ (1, 5); phosphatidylinositol 3-kinase PI4K92; indeed, the antigen-antibody complex is catalytically active (5). What might be the function of these polyphosphoinositides on the speckles as well.

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Addendum—During preparation of this manuscript, an article was published by A. Hausser et al (41) reporting the presence of a Ser-294 phosphorylated form of PI4K92 (PI4KIIIβ) overexpressed in Cos7 cells on the Golgi complex. Phosphorylation was catalyzed by PKD1 and PKD2. Additionally, an anti-pSer-294 phosphopeptide antibody against an epitope comprising amino acids 289–297, slightly modified (K297L), precipitated PI4K92 from HEK293 cells. Thus, their conclusions are in perfect agreement with the observation reported here.

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