Phosphatidylinositol 3-kinase C2α contains a nuclear localization sequence and associates with nuclear speckles

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ABSTRACT

Phosphoinositide 3-kinase C2α (HsPI3K-C2α) belongs to the class II phosphatidylinositol 3-kinases (PI 3-kinases) which are defined by their in vitro usage of phosphatidylinositol (PtdIns) and PtdIns 4-phosphate (PtdIns(4)P) as substrates. All type II PI 3-kinases contain at their carboxy-terminus a C2-like domain. Here we demonstrate that HsPI3K-C2α has dual cellular localization being present in the cytoplasm and in the nucleus. A distinct nuclear localization signal (NLS) sequence was identified by expressing HsPI3K-C2α–GFP fusion proteins in HeLa cells. The NLS was mapped to a stretch of 11 amino acids (KRKTKISRKTR) located within C2-like domain of the kinase. In the cytoplasm and the nucleus HsPI3K-C2α associates with macromolecular complexes which are resistant to detergent extraction. Indirect immunofluorescence reveals that in the nucleus HsPI3K-C2α is enriched at distinct subnuclear domains, known as nuclear speckles, which contain pre-mRNA processing factors and are functionally connected to RNA metabolism. Phosphorylation of HsPI3K-C2α is induced by inhibition of RNA polymerase II-dependent transcription and coincides with enlargement and rounding up of the nuclear speckles. The results suggest that phosphorylation of HsPI3K-C2α is inversely linked to mRNA transcription and support the importance of phosphoinositides for nuclear activity.
INTRODUCTION

Phosphatidylinositol 3-kinases (PI 3-kinases) have emerged as important constituents of cellular pathways regulating the remodeling of the cytoskeleton, the trafficking of intracellular organelles and cell growth and survival (1). Type I PI 3-kinases are primarily involved in receptor mediated signal transduction and preferably use PtdIns(4,5)P₂ as substrate in vivo, but phosphorylate also phosphatidylinositol and phosphatidylinositol 4-phosphate (PtdIns(4)P) at the 3-OH position of the inositol ring. The type III PI 3-kinases exclusively phosphorylate PtdIns and are essential for vesicular trafficking. Both type I and type III PI 3-kinases are heterodimeric enzymes consisting of a catalytic and a regulatory subunit. By contrast, the type II PI 3-kinases are monomeric enzymes, which use phosphatidylinositol (PtdIns) and PtdIns 4-phosphate (PtdIns(4)P) as substrates. Their function in signal transduction is poorly understood. Few studies provided evidence for a role of type II kinases in chemokine and growth factor receptor mediated cell activation (2-6) and in integrin mediated platelet activation (7).

A common structural characteristic of type II PI 3-kinases is a C2-like domain at the C-terminus. The C2-domains of many proteins mediate calcium dependent phospholipid binding (8). However type II PI 3-kinases lack a critical aspartate residue in their C2-like domain and the requirement of calcium for membrane binding and catalytical activity remains controversial (4;9). Three human type II PI 3-kinases have been characterized: HsPI3K-C₂α, β and γ. They share a similar structure containing an unique N-terminus, a catalytic domain, a PX-domain and a C2-like domain at the C-terminus (10). While HsPI3K-Cα and HsPI3K-Cβ share wide tissue distribution, HsPI3K-Cγ expression is restricted to hepatocytes and is enhanced during liver regeneration (11;12). Both HsPI3K-Cα and HsPI3K-Cβ are implicated in signaling downstream of EGF and PDGF receptors (4). Signaling through insulin receptor or chemokine receptors induces activation of HsIP3K-C₂α (2;5). Recent reports demonstrate that HsIP3K-
C2α is concentrated in trans-Golgi network and present in clathrin-coated pits (13), whereas PI3K-Cβ was found in the nuclei of rat liver cells (6).

Phosphoinositide signaling in the nucleus is regulated independently from plasma membrane phosphoinositide pathways. Some nuclear phosphoinositides and their metabolizing enzymes are not extracted with non-ionic detergents which indicates that they are not associated with membrane structures (14;15). Two PtsIns(4)P 5-kinases (PIPKIα and PIPKIIα) are reported to localize to nuclear speckles together with their product PI(4,5)P2 (15). By electron microscopy analysis, speckles were found to consist of two morphologically and functionally distinct domains. The larger and denser regions seen by fluorescence microscopy correspond to interchromatin granule clusters, which are not active in transcription. The more diffusely distributed splicing factors and the regions of the periphery of the interchromatin granule clusters correspond to perichromatin fibrils, where transcription takes place (16-18). The composition of nuclear speckles is highly dynamic, because pre-mRNA transcribing and processing factors move rapidly in and out and are concentrated by transient associations with functionally related components (19). Thus, the overall morphological appearance of nuclear speckles reflects the transcriptional activity of the cell (20). In response to inhibition of RNA polymerase II-dependent transcription speckles become larger, round up and lose their irregular shape (21-23). Furthermore, it has been proposed that cycles of phosphorylation and dephosphorylation control the subnuclear distribution of splicing factors thereby regulating their association with the speckles (24;25).

We show here that HsPI3K-C2α is present in the cytoplasm and in the nucleus. In many cases translocation of proteins into the nucleus is conferred by distinct import signals. The best characterized are nuclear localization signals (NLS) consisting of one or more clusters of basic amino acids (26). A signal sequence for nuclear localization of HsPI3K-C2α was mapped to a short stretch of highly basic amino acids localized within the C2 domain of the kinase, a domain
that is known to target a variety of proteins to the plasma membrane. In the nucleus the HsPI3K-Cα concentrates at nuclear speckles together with pre-mRNA processing factors.
EXPERIMENTAL PROCEDURES

Antibodies against PI3K-C2 were raised in rabbits immunized with chimeric proteins containing glutathione S-transferase (GST) fused either to amino acids 62-131 of murine p170 (Accession: U55772) (27) or to the N-terminus (amino acids 1-134) of HsPI3K-C2α (Accession: Y13367) (28). Immune sera were purified by affinity chromatography using the corresponding domains of p170 (antibody AXIX) and HsPI3K-C2α (antibody AXXIII) immobilized on NHS-sepharose (APB). Briefly, isolated GST fusion proteins were proteolysed with thrombin and the cleaved products further purified by reverse phase high pressure liquid chromatography. Anti-lamin goat polyclonal IgG was purchased from Santa Cruz Biotechnology, anti-GFP rabbit polyclonal antibody was from Clontech, horseradish peroxidase (HRP)-conjugated goat anti-rabbit from Bio-Rad. Fluorescent dye-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Human autoimmune Sm serum and anti-CAP mAb were kindly provided by Dr. A. Rosen (Baltimore, MD) and Dr. R. Luhrmann (Göttingen, FRG), respectively.

Plasmids - The cDNA encoding human HsPI3K-C2α (28) was a kindly provided by Dr. J. Domin (London, UK). DNA fragments corresponding to subdomains of HsPI3K-C2α were generated by the polymerase chain reaction (PCR) using specific primers. For the transient expression the fragments were cloned into the eukaryotic expression vector pEGFP (Clontech). Domains corresponding to the N-terminus of HsPI3K-C2α were fused upstream of GFP (vector pEGFP-N1), whereas domains corresponding to the C-terminus of HsPI3K-C2α were fused downstream of GFP (vector pEGFP-C1). Parts of the C2-like domain of HsPI3K-C2α were fused to the C-terminus of red fluorescent protein (vector pDsRed1-C1, Clontech).

Cell culture and transient expressions - HeLa (ATCC) cells were cultured at 37°C in Dulbecco’s modified Eagle minimal essential medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) and antibiotics. Treatment of HeLa cells with 5 μg/ml actinomycin D,
50 µg/ml cycloheximide or 40 µg/ml α–amanitin (all from Sigma) was carried out for 5h at 37°C. For transfection, subconfluent cells cultures were trypsinized, washed and resuspended at 2.5 x 10⁶ cells/ml in DMEM containing 10 % FCS and 15 mM HEPES, pH 7.5. To 200 µl cell suspension 50 µl of DNA mixture (200 mM NaCl, 10 µg of plasmid DNA and 30 µg of carrier DNA (salmon sperm, Stratagene)) was added. After electroporation (960 µF, 240 V) the cells were cultured for 16-48 hours before analysis.

Subcellular fractionation - HeLa cells (~ 1-5 x 10⁷ cells) were treated with cytochalasin B (10 µg/ml) in culture medium for 30 min, trypsinized, washed two times with PBS and resuspended in 1 ml of ice cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 10 µg/ml cytochalasin B, 40 mM NaF, 0.5 mM sodium orthovanadate, 40 mM β–glycerophosphate, 5 mM sodium pyrophosphate and protease inhibitors (Complete, Roche)). Cells were allowed to swell on ice for 10 min and then passed several times through a 27 gauge syringe needle. The cell homogenate was layered onto 300 µl of 30 % sucrose (wt/vol) in lysis buffer and spun at 2'000 g for 10 min. The postnuclear supernatant (PNS) containing membranes and cytosol was collected and further processed (see below). The pellet consisting of nuclei and few unbroken cells was resuspended in lysis buffer, homogenized, divided into four equal parts and centrifuged as before. Following an additional wash with lysis buffer nuclear pellets were resuspended either in lysis buffer and left on ice for 15 min, or in lysis buffer containing 1 % Triton X-100 and placed for 15 min on ice, or in high salt buffer (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1mM EDTA, 1 mM DTT) and incubated on ice for 30 min, or in 10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 1.5 mM MgCl₂, containing RNase free DNase I (20 U/ml, Roche) and incubated for 20 min on ice. The aliquots were then centrifuged at 2'000 g for 10 min. Proteins in the supernatants and pellets were precipitated with 10 % trichloroacetic acid (TCA), washed with cold acetone, and finally dissolved in SDS sample buffer (60 mM Tris-HCl (pH 6.8), 10 %
glycerol, 2 % SDS, 100 mM DTT, 0.01 % bromphenol blue), boiled for 5 min and analysed on SDS-polyacrylamide gels as described below.

Nuclear envelopes were separated from the nuclear extracts as follows. Nuclei were resuspended in buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl, 3 mM MgCl₂, 0.1 mM EDTA, 8 U/ml RNAsin (Promega), 40 mM NaF, 0.5 mM NaVO₄, 40 mM β-glycerophosphate, 5 mM sodium pyrophosphate and protease inhibitors (Complete, Roche)) and disrupted by sonication using a micro-tip (two 15-sec pulses at 50W). The homogenate was centrifuged for 30 min at 10'000 g to pellet nuclear envelopes, and the supernatant (nuclear extract) was further separated by high speed centrifugation (15 min, 400'000 g) into a soluble fraction and a pellet.

The PNS was fractionated into cytosol and a high speed pellet consisting of membranes, cytoskeleton and ribosomal structures as follows. PNS was passed three times through a 27 gauge needle and cleared at 5'000 g for 15 min, and the resulting supernatant was centrifuged at 400'000 g for 10 min at 4° C.

For RNAse treatment high speed pellets prepared from PNS and nuclear extract were resuspended in RNAse digestion buffer (10 mM Tris-HCl (pH 8.0), 1 % 2-mercaptoethanol) and incubated without or with RNAases A (1 mg / ml ) and T1 (50 U / ml) at 37° C for 30 min. After treatment the samples were fractionated into supernatant and pellet at 400'000 g for 15 min.

**Gel electrophoresis, immunoprecipitation and Western blot analysis** - Proteins were separated on 8 % or 6 % SDS-polyacrylamide gels prepared from a acrylamide stock (33.5 % acrylamide : 0.3 % bis acrylamide) and blotted onto Immobilon-P (Millipore). Membranes were blocked with 5% non-fat milk in TBS containing 0.1% Triton X-100, and probed with specific antibodies. Immunoreactive bands were decorated with horseradish peroxidase-labeled antibodies or protein G-conjugated with horseradish peroxidase (Zymed) and visualized by enhanced chemiluminescence (Pierce).
For immunoprecipitation, cells were washed twice in PBS and lysed in buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, 1mM EDTA), supplemented with phosphatase inhibitors (40 mM NaF, 0.5 mM sodium orthovanadate, 40 mM β–glycerophosphate, 5mM sodium pyrophosphate) and protease inhibitors (Complete, Roche). Cell homogenates were centrifuged at 13'000 g for 10 min, and supernatants were precleared with GammaBind G-Sepharose (APB) for 15 min. Immunoprecipitation of HsPI3K-C2α with antibody AXXIII was carried out at 4° C for 1-2 h. Immune complexes were bound to GammaBind G Sepharose for 30 min., collected by centrifugation and washed two times in lysis buffer, once in 10 mM Tris-HCl (pH 8), 0.5M NaCl, 0.5 % NP-40, 0.5 % deoxycholate, 0.05 % SDS; then in 10 mM Tris-HCl (pH 8), once in 10 mM Tris-HCl (pH 8.), 150 mM NaCl, 0.5 % NP-40, 0.5 % deoxycholate, 0.05 % SDS, and finally in 10 mM Tris-HCl (pH 8), 0.05 % SDS.

For λ–phosphatase treatment immunoprecipitates were additionally washed twice in phosphatase buffer (50 mM Tris-HCl (pH 7.5), 2 mM MnCl₂, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35) and resuspended in 50 µl of the same buffer. After warming up at 30° C for 3 min., 50 U of λ–phosphatase (New England Biolabs) was added and the samples were incubated at 30° C for 40 min.

Kinase assay – HsPI3K-C2α was immunoprecipitated as described in the previous section except that the washing steps were omitted, instead immune complexes bound to GammaBind were washed three time with lysis buffer and than treated with buffer or λ–phosphatase as mentioned above. Following phosphatase treatment immunoprecipitates were washed twice with 0.5 M LiCl, 50 mM Tris-HCl (pH 8.0), once with 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and once with 20 mM Hepes (pH 7.3), 1 mM DTT, 5 mM MgCl₂.

Resulting immunoprecipitates were resuspended in kinase assay buffer (50mM Tris-HCl (pH 7.6), 50 mM NaCl, 20 mM glycerophosphate, 0.1mM Na-Vanadate, 10 mM NaF).

PI 3-kinase activity was determined in of 50 µl kinase assay buffer containing 10μg each PtdIns and phosphatidyl serine, 0.1% Na-Cholate, 0.25 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂.
and 0.1 mM ATP. Samples were warmed up for 10 min at 30°C and reactions initiated by the addition of MgCl₂ and ATP (containing 5-10 µCi [³²P]γATP (Amersham Pharmacia Biotech)). Reactions were terminated after 20 min with 100 µl 1M HCl, and the lipids extracted with 200 µl chloroform:methanol (1:1). The aqueous phase was washed twice with 80 µl 1M HCl:methanol (1:1), the lipids were dried and analyzed by thin layer chromatography as described (29).

Immunofluorescence - HeLa cells, grown on glass coverslips, were permeabilized with 1% Triton X-100, 2 mM EGTA, 5 mM Pipes (pH 6.7) for 1 min at room temperature and immediately fixed in cold (-70°C) methanol for 10 min or in 0.4% PFA for 20 min at room temperature. After several washes in PBS the coverslips were blocked in PGB (PBS containing 10% goat serum (Sigma) and 0.5% BSA (Sigma)) for 15 min followed by the incubation with the primary antibody for 1 h in a humidified chamber. Antibodies were diluted in PGB and used at the following concentrations: affinity-purified anti-HsPI3K-C2α (5 µg/ml), anti-Sm serum (1:1000 dilution). The coverslips were then washed several times with PBS and incubated again with PGB for 10 min. Fluorescence dye conjugated secondary antibodies (1 µg/ml) were added for 1 hour in PGB. Coverslips were then washed extensively with PBS and once with water, and mounted in polyvinyl alcohol (Gelvatol, Sigma) supplemented with 1% DABCO (Sigma).
RESULTS

*Nuclear an cytoplasmic localization of HsPI3K-C2α in resting cells* – PI 3-kinases were shown to associate with different cellular compartments including the cytosol, the plasma membrane, endosomes and the nucleus. To study the subcellular localization of the type II HsPI3K-C2α we generated two polyclonal antibodies directed against the N-terminus of the human sequence (amino acids 1-134, AXXIII) and a domain encompassing amino acids 62-171 of the murine homologue p170 (AXIX). Both antibodies immunoprecipitated HsPI3K-C2α from cell lysates and recognized the protein on Western blots. The specificity of the antibodies was further confirmed by mass spectrometry of peptides obtained by proteolytic digestion of immunoprecipitated proteins (Table 1). Affinity purified antibody preparations were used for the indirect immunofluorescence analysis of the subcellular localization of HsPI3K-C2α. As shown in Fig. 1, HeLa cells stained with antibody AXIX display a weak punctated staining in the cytoplasm. The most prominent signal was observed in the nucleus where HsPI3K-C2α concentrates in speckle-like structures. HsPI3K-C2α-positive speckles appear to surround nucleoli and were not observed outside of the nucleus. This particular nuclear localization of HsPI3K-C2α was also detected in primary human dendritic cells and a variety of cell lines tested, such as human pancreatic Aspc-1 cells and epithelial tumor MCF7 cells, as well as murine macrophages J774A.1 and fibroblasts NIH-3T3. Similar immunostaining pattern of HsPI3K-C2α is observed with antibody (AXXIII) with the only difference that the speckles were set against a diffuse staining of the nucleoplasm. The difference is most likely due to the accessibility of the distinct epitopes. Antibody AXXIII recognizes HsPI3K-C2α which is associated with speckles and diffusely distributed in the nucleoplasm, whereas antibody AXIX reacts preferentially with a subset of HsPI3K-C2α that is present at speckles. Preincubation of antibodies with the corresponding recombinant antigens completely abolished nuclear staining (not shown). The staining of HsPI3K-C2α was independent on the fixation method employed,
however, the best results were obtained by using methanol fixation. Permeabilization of cells with Triton X-100 prior to fixation did not alter the nuclear staining pattern, indicating that the kinase was resistant to detergent extraction.

Cell fractionation experiments were performed to further investigate the subcellular localization of HsPI3K-C2α. Homogenates of HeLa cells were subjected to differential centrifugation and separated into nuclei and postnuclear supernatant (PNS). Fig. 2A shows that HsPI3K-C2α is present in the nuclear fraction and in the PNS. Following high speed centrifugation of the PNS, all HsPI3K-C2α was recovered in the pellet from which it could not be solubilized with the detergent Triton X-100. Treatment of nuclei with the Triton X-100, which removes nuclear membranes but leaves the nuclear lamina intact, also did not release HsPI3K-C2α (Fig. 2A). In accordance with the immunofluorescence data these findings indicate that in the nuclei and in the PNS HsPI3K-C2α is not retained by cellular membranes but it is associated with Triton X-100-insoluble macromolecular structures. Extraction of nuclei with high ionic strength (0.4 M NaCl) or treatment with DNAse I also did not solubilize HsPI3K-C2α (Fig. 2A), suggesting that the kinase is not bound to DNA, but rather associates with the nuclear matrix or RNA containing structures. Fractionation of the nuclei into nuclear extract and envelope resulted in separation of HsPI3K-C2α from lamin B, which suggests that the kinase is not a constituent of the nuclear envelope (Fig. 2B). High speed centrifugation led to the precipitation of HsPI3K-C2α from nuclear extract indicating that in the extract the kinase remained associated with macromolecular structures. Next, we examined whether the association of HsPI3K-C2α with nuclear and cytoplasmic complexes depends on the presence of RNA. Fig. 2C shows that after prolonged incubation with RNases A and T1 HsPI3K-C2α was almost completely released from the high speed pellet obtained from nuclear extracts and also to a large extent released from complexes isolated from PNS. From this finding it can be concluded that in both the cytoplasm and the nucleus, HsPI3K-C2α associates with RNA containing structures.
HsPI3K-C2α localizes to nuclear speckles together with mRNA-processing factors - Many nuclear factors localize in distinct nuclear 'bodies' or subnuclear compartments that exhibit a spot like pattern when analyzed by indirect immunofluorescence microscopy. Because HsPI3K-C2α shows a comparable staining pattern, we examined with which type of subnuclear bodies the kinase associates. Double labeling of HeLa cells with antibody AXIX and a human autoimmune Sm antiserum, that reacts with Sm proteins and U170K of snRNPs, revealed a marked co-localization HsPI3K-C2α with snRNPs (Fig. 3). Many Sm antiserum reactive speckles were also positive for HsPI3K-C2α. A similar pattern of co-localization was observed for HsPI3K-C2α and 5’-terminal trimethylguanosine (m3G) cap of snRNPs (not shown). However, double immunostaining of HeLa cells for HsPI3K-C2α and known components of POD (PML organizing domains containing the PML protein), coiled bodies (fibrillarin, coilin A) and particles containing the autoantigen of Sjorgen’s syndrome La/SS-B failed to show an association of the kinase with these nuclear structures (not shown). Thus, HsPI3K-C2α displays significant co-localization only with snRNPs.

High transcriptional activity of the cell coincides with a more widespread localization of pre-mRNA processing factors and small speckles, whereas upon inhibition of transcription by RNA polymerase II speckles lose their irregular shape, round up, and fuse into larger clusters. To examine the effect of the inhibition of RNA polymerase II on the subnuclear localization of HsPI3K-C2α, HeLa cells were treated with α-amanitin (40 µg/ml) or actinomycin D (5 µg/ml) for 5h. Cells incubated with α-amanitin showed a similar alteration in the shape of HsPI3K-C2α-positive and Sm-positive speckles (Fig. 3). Speckles appear more round and connections between them are no longer visible compared with control cells (Fig.3). The same effect was observed when cells were treated with actinomycin D (not shown). By contrast, inhibition of translation with cycloheximide (50 µg/ml) for 5 h had no effect on the subnuclear localization of HsPI3K-C2α and Sm proteins (Fig. 3). This observation indicates that the structure of the
Nuclear localization of HsPI3K-C2α

Didichenko et al., page 14

speckles is not maintained by labile protein factors. Inhibition of transcription or translation neither caused a redistribution of HsPI3K-C2α between nucleus and cytoplasm nor induced any changes in its expression level as measured by Western blot analysis (Fig. 4A). However, in cells treated with α-amanitin or actinomycin D a substantial fraction of HsPI3K-C2α migrates on SDS-gels with slower electrophoretic mobility (Fig. 4). Retarded electrophoretic mobility is observed for both, cytosolic and nuclear proteins. This effect was barely detected in samples obtained from control or cycloheximide treated cells. Thus, upon inhibition of transcription HsPI3K-C2α might undergo post-translational modifications, such as phosphorylation.

Consistent with this, phosphatase treatment of HsPI3K-C2α immunoprecipitates abolished the appearance of the slower migrating band (Fig. 4). Phosphorylation of HsPI3K-C2α appears not to affect the catalytic activity. Figure 4c shows that HsPI3K-C2α immunoprecipitated from control and actinomycin D treated cells possesses similar activity and that the activity was not altered upon phosphatase treatment. Taken together these observations demonstrate that the distribution of HsPI3K-C2α strongly correlates with the localization of components of the splicing apparatus and depends on the transcriptional activity of the cell. Furthermore, the subnuclear distribution of HsPI3K-C2α appears to be linked to its state of phosphorylation.

The C2-like domain of HsPI3K-C2α contains a nuclear localization sequence- The biochemical and immunofluorescence data indicated that HsPI3K-C2α is present in the nucleus. We therefore searched for sequence elements that could confer nuclear translocation of the protein. Chimeric proteins composed of different segments of HsPI3K-C2α and green fluorescent protein (GFP) were transiently expressed in HeLa cells (Fig. 5). All constructs covering the N-terminus and the catalytic domain of HsPI3K-C2α (1-275/GFP, 1-482/GFP, 474-972/GFP, and GFP/649-1545) reveal strictly cytoplasmic localization (Fig. 5A and B). By contrast, a chimeric protein containing the entire C2-like domain (GFP/649-1658 and GFP/1204-1658) localizes in the cytosol and in the nucleus. Further truncation of the N-terminus including a part of the C2-like
domain led to a fusion protein that shows predominant nuclear localization (GFP/1546-1658). This suggested that the C2-like domain of HsPI3K-C2α includes a nuclear localization sequence (NLS). The NLS was mapped within C2-like domain by expressing additional deletion variants. Only those fusion proteins accumulated in the nucleus, which contained a short stretch of 11 mostly basic amino acids (KRKTKISRKTTR) located at amino acids 1608 to 1619 near the C-terminus of HsPI3K-C2α (Fig. 5). This peptide fused either to GFP or red fluorescent protein (DsRed) was sufficient to direct both chimeric proteins to the nucleus (Fig. 5C). All fusion proteins which contain the NLS show a preference for accumulation in the nucleoli. This is in contrast to the endogenous HsPI3K-C2α which appears to localize to the nuclear speckles and to distribute around nucleoli (Fig. 1). This difference suggests that in addition to the NLS other sequence elements are necessary for the distinct localization of HsPI3K-C2α at nuclear speckles. The chimeric protein (GFP/649-1568) which encompasses the catalytic domain and the entire C2 domain reveals nuclear localization but fails to show an association with speckles (Fig. 5), suggesting that the catalytic domain does not mediate subnuclear localization.
DISCUSSION

In this study we demonstrate that in resting cells the type II PI 3-kinase HsPI3K-C2α resides in the cytoplasm and in the nucleus. Recently it has been shown that the structurally related PI3K-C2β is associated with membrane depleted nuclei of rat liver cells (6). These observations reveal a remarkable difference with the localization of type I PI 3-kinases, which are generally considered cytosolic proteins (1;30;31). Few reports provide evidence for type IA PI 3-kinase activity in the nuclei of stimulated cells, such as human osteosarcoma (32) and rat liver cells (33). The catalytic subunit of type IB PI 3-kinaseγ has recently been demonstrated to translocate to the nuclei of HepG2 cells after stimulation with serum (34). However, the mechanism of translocation and activation of PI3-kinases in the nucleus is poorly understood.

Our data provide evidence that HsPI3K-C2α concentrates in the interchromatin granule clusters (nuclear speckles). Nuclear speckles are known to contain factors involved in the transcription and processing of pre-mRNA including RNA polymerase II, snRNPs and non snRNP splicing factors (35). Splicing factors associated with speckles are resistant to extraction with non-ionic detergent, high salt, or treatment with DNAse I, suggesting that speckles represent nuclear compartments which are possibly connected with the nuclear scaffold (36;37). We show that HsPI3K-C2α exhibits all these properties common to splicing factors. The overall morphological appearance of nuclear speckles is indicative for the transcriptional activity of the cell. Upon inhibition of RNA polymerase II the speckles lose their irregular shape and collapse into round and larger clusters (36;38). HsPI3K-C2α was found to redistribute identically with other speckle components (Sm-antigens) in response to inhibition of RNA II polymerase by α–amanitin (Fig. 3). The observation suggests that HsPI3K-C2α directly interacts with some components of nuclear speckles. It is conceivable that the kinase is in a complex with some type of RNA because treatment with RNAses leads to its solubilisation (Fig. 2C). A likely candidate for the interaction is poly(A)^+ mRNA, since HsPI3K-C2α was found in preparations of poly
mRNPs which were purified by affinity chromatography on oligo(dT)-cellulose (our unpublished results). We could not detect HsPI3K-C2α in snRNP preparations, and in vitro splicing assays appeared to be insensitive to the PI 3-kinase inhibitor wortmannin (R. Lurhman, personal communication). Therefore, the direct interaction of HsPI3K-C2α with snRNP splicing factors is less probable.

The reversible phosphorylation of splicing factors on serine/threonine residues is known to affect their localization in nuclear speckles and regulate their activity (24,25,39). We found that inhibition of transcription which causes the collapse of nuclear speckles is accompanied by the phosphorylation of HsPI3K-C2α. It is, therefore, plausible that the state of phosphorylation is critical for the subnuclear localization of HsPI3K-C2α. In agreement with the continued association with nuclear speckles, we did not obtain an evidence that phosphorylation of HsPI3K-C2α causes redistribution of the kinase between the nucleus and the cytosol. In the cytosol phosphorylation of HsPI3K-C2α did not cause a detectable change of the immunostaining pattern. Preliminary data suggests that the phosphorylation of HsPI3K-C2α induced by inhibition of transcription occurs at serine residues. Protein kinases, SRPK-1 (25), CLK/STY (39) and casein kinase Iα (40) localize to nuclear speckles and phosphorylate non-snRNP splicing factors (SR-proteins). Nonetheless, it is unlikely that HsPI3K-C2α is phosphorylated by either kinase since HsPI3K-C2α lacks the serine/arginine rich consensus essential for phosphorylation by SRPK-1 and CLK/STY, and also does not contain an appropriate consensus sequence for casein kinase Iα. Identification of the kinase which phosphorylate HsPI3K-C2α may provide an insight on the regulation of the HsPI3K-C2α.

We found a similar subcellular distribution of endogenous HsPI3K-C2α in cells from different tissues including human primary dendritic cells, mesenchymal cells (HeLa), pancreatic carcinomas (Aspc-1, T3M4) and epithelial tumor cells (A549) as well as murine macrophages (J7741.A) and fibroblasts (NIH3T3). Our immunofluorescence data do not provide the evidence
that HsP13K-C2α associates with the trans Golgi network. This contrasts somehow with a previous report by Domin et al. who showed by indirect immunofluorescence that in HEK293 cells HsP13K-C2α co-localizes with γ-adaptin (13). Accessibility of the HsP13K-C2α for different antibodies and differences in the fixation protocols used during immunofluorescence staining could be the reason for the discrepancy. However, we observed a similar nuclear localization of HsP13K-C2α in non-fixed HeLa cells which were just permeabilized with Triton X-100 before staining (not shown). This particular method has been applied to reveal the dynamic distribution of pre-mRNA splicing factors localized to nuclear speckles (41).

In the cytoplasm HsP13K-C2α appears to be tightly associated with macromolecular complexes and also to be resistant to solubilization with detergent, indicating that the kinase is not retained by membranes. The biochemical composition of the cytoplasmic complexes remains to be resolved. The fact that RNase treatment only leads to partial solubilisation of HsP13K-C2α from cytoplasmic complexes (Fig. 2C) argues that the in the cytoplasm HsP13K-C2α resides in two distinct pools. One pool appears to be associated with RNA while the other could be indicative for HsP13K-C2α bound to clathrin-coated vesicles (13). It less probable that HsP13K-C2α associates with the actin cytoskeleton or microtubules, since treatment of cells with the actin depolymerizing compound cytochalasin D or microtubule destabilizing conditions did not cause its solubilization (unpublished observations).

Localization of HsP13K-C2α to nuclear speckles implies some mechanism of nuclear import. A prerequisite for many proteins to be imported into the nucleus is a NLS. By expressing different segments of HsP13K-C2α as GFP-fusion proteins, we identified the highly basic sequence KRKTKISRKTR to be the determinant for the nuclear localization of HsP13K-C2α. This sequence which is located within the C2-like domain contrasts the function of known C2-domains which target proteins to the plasma membrane (42). The NLS of HsP13K-C2α does not
show strong similarity to other known nuclear targeting sequences, rather its overall composition of basic (7 out of 11) amino acids provides some degree of homology (Table 1). The best similarity was found with the NLS of rat ribosomal protein L31 (RLSRKR) (43). The homology with the NLS of ribosomal protein may explain the pronounced nucleolar localization of HsPI3K-C2α-NLS-GFP and HsPI3K-C2α-NLS-DsRed fusion proteins (Fig. 5C). The observation that the endogenous HsPI3K-C2α is not present in the nucleoli suggests that additional domains are required to target the protein to the nuclear speckles. Comparison of type II PI3-kinases from different species revealed that the sequence KRKTISRKTR is conserved in HsPI3K-C2α and the murine PI3K-C2 kinases, p170-m and cpk-m (Table 1). This is in an agreement with our observation that in murine J7741.A and NIH 3T3 cell lines cpk-m localizes to nuclei. The human isoforms HsPI3K-C2β and HsPI3K-C2γ show partial homology in the region of NLS, however, only the tetrapeptide RKTK is fully conserved (Table 1). It remains an open question whether the corresponding sequences present in HsPI3K-C2β and HsPI3K-C2γ cause nuclear targeting. The fact that PI3K-C2β was found in nuclei of rat liver cells (6) and the observation that rat PI3K-C2γ overexpressed in COS cells shows perinuclear localization (12) suggests that translocation of different PI3K-C2 isoforms to the nucleus is mediated by the homologous sequences.

The remarkable nuclear localization of HsPI3K-C2α suggests that phosphoinositides produced at speckles are critical regulators of nuclear activities. Two other PtdIns phosphate 5-kinases (PIPK), the type I and II isoforms, which exhibit different substrate specificity, were also shown to associate with nuclear speckles (15). Upon inhibition of transcription PIPKs redistribute identically with their product PtdIns(4,5)P2 and defined speckle components. The hypothesis that nuclear polyphosphoinositides are involved in regulation of transcription is further supported by the observation that the negatively charged lipids PtdIns(4,5)P2 and PtdIns(3,4,5)P3 can reverse the inhibition of transcription caused by histone H1 (44) and that
PtdIns(4,5)P₂ is involved in BAF-mediated chromatin remodeling (45). A specific role for PI(3,4)P₂, the major product of HsPI3K-C2α, in nuclear events remains to be shown.

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**REFERENCES**


FIGURE LEGENDS

Fig. 1. HsPI3K-C2α is concentrated in nuclear speckles. HeLa cells were fixed with cold methanol and stained with affinity purified anti-HsPI3K-C2α antibody AXIX. Nuclei were stained either with DAPI or propidium iodide (PI).

Fig. 2. Subcellular distribution of HsPIK-C2α. (A) Cell lysate (lysate) was prepared from HeLa cells and fractionated into nuclear pellet (total) and postnuclear supernatant (PNS) by low-speed centrifugation. Aliquots of nuclei were incubated in the presence of 1 % Triton X-100, 0.4 M NaCl, or DNase I (20 U/ml). Following incubation the samples were fractionated into the supernatant (S) and the pellet (P). PNS was separated into membrane pellet (P) and cytosol (S) by high-speed centrifugation. The membrane pellet was extracted with 1 % Triton X-100 and separated into a pellet (P) and a supernatant (S) as above. Aliquots from each fraction were analysed on Western blots using anti-HsPIK-C2α antibody AXIX and anti-lamin B antibody. (B) Subnuclear distribution of HsPIK-C2α. Nuclei (total) prepared from HeLa cells were disrupted by sonication and fractionated into nuclear extract (extract) and envelopes (envelope). Nuclear extract was further separated into soluble (S) and insoluble (P) fractions by high-speed centrifugation. (C) Treatment with RNAse leads to solubilization of HsPI3K-C2α from macromolecular complexes. High-speed pellets prepared from postnuclear supernatant (PNS-P) and nuclear extract (NE-P) were resuspended in RNAse digestion buffer and incubated in the absence (-) or presence (+) of RNAeses A and T1. After incubation samples were
centrifuged at high speed, and proteins in the resulting supernatant (S) and pellet (P) were analysed on Western blots.

Fig. 3. HsPI3K-C2α co-localizes with snRNP at nuclear speckles. Effect of inhibition of transcription on its nuclear localisation. HeLa cells were incubated for 5 h in the absence (control) or presence of α-amanitin (40 µg/ml) or cycloheximide (50 µg/ml). Cells were fixed with methanol and double stained for HsPI3K-C2α and snRNP using affinity purified anti-HsPI3K-C2α antibody AXIX and human Sm antiserum. HsPI3K-C2α shows strong co-localization with snRNP. Treatment with α-amanitin causes identical rearrangement of HsPI3K-C2α and Sm antigens. The translation inhibitor cycloheximide has no effect on the nuclear distribution of HsPI3K-C2α.

Fig. 4. Inhibition of transcription leads to phosphorylation of HsPI3K-C2α. HeLa cells were treated for 5 h with the α-amanitin (40 µg/ml), actinomycin D (5 µg/ml), or cycloheximide (50 µg/ml). (A) Nuclear (N) and cytosolic (C) fractions were prepared and subjected to Western analysis. (B) Immunoprecipitates of HsPI3K-C2α were treated with λ-phosphatase (+λPPase) or buffer alone (-λPPase) for 40 min and analysed as above. (C) Kinase activity assays were performed in immunoprecipitates prepared from control and actinomycin D treated cells. Where indicated, immunoprecipitates were pretreated with λ-phosphatase (+λPPase).

Fig. 5. The C2 domain of HsPI3K-C2α contains a nuclear localisation sequence. (A) Structure of HsPI3K-C2α and schematic representation of HsPI3K-C2α-GFP fusion proteins. Parts of HsPI3K-C2 are indicated by a black box, GFP - by a green oval, DsRED – by a red box. Localization of the expressed fusion proteins in HeLa cells is indicated on the right. C, cytoplasm; N, nucleus. (B) GFP-HsPI3K-C2α fusion proteins were transiently expressed in HeLa cells and their subcellular distribution was determined by indirect immunofluorescence.
with an anti-GFP antibody. (C) Intracellular distribution of GFP-KRKKTKISRKTR and DsRED-KRKKTKISRKTR fusion proteins.
**TABLE 1**

Proteolytic peptides of HsPI3K-C2α detected by mass spectrometry

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Mass Determination</th>
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<tr>
<td>D45RQVDNGRGEFLSSSTRK63</td>
<td>MALDI-TOF</td>
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<tr>
<td>R86ALIDVEK94</td>
<td></td>
</tr>
<tr>
<td>I230ASTSEFLK238</td>
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<td>D1016ALHDVQSFSTRYEHVLSVGGK1040</td>
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<td>R1041LREELK1048</td>
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<td>T1415YSFRQDGRK1425</td>
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</tbody>
</table>

Peptides were obtained after digestion with endoproteinase lys C. Mass determination was performed by MALDI-TOF.
## TABLE 2

### Nuclear localization sequences

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<th>Sequence</th>
<th>Consensus</th>
<th>References</th>
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<tr>
<td>Bipartite domain of nucleoplasmin</td>
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</tr>
<tr>
<td>SV40 large T-antigen</td>
<td>PKKRRKV</td>
<td>(48)</td>
</tr>
<tr>
<td>Ribosomal protein L31</td>
<td>RLSRKR</td>
<td>(43)</td>
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<td>this study</td>
</tr>
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<td>potential NLS</td>
</tr>
<tr>
<td>HsPI3K-C2γ</td>
<td>RRKTKSVPTK</td>
<td>potential NLS</td>
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<tr>
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<tr>
<td>S6</td>
<td>KRRRIALKKQRTKKNK</td>
<td>(52)</td>
</tr>
</tbody>
</table>
Figure 1

HsPI3K-C2α

Prop. iod.

DAPI
Figure 3

HsPI3K-C2α | Sm
---|---
control
α-amanitin
cycloheximide
Figure 4

A

C N C N C N C N
control -amanitin actinomycin D cycloheximide

B

- PPase PPase

control -amanitin actinomycin D cycloheximide control -amanitin actinomycin D cycloheximide

C

- PPase PPase - PPase PPase

control actinomycin D
Figure 5

A

HsPI3K-C2α

Localization

C    N

-    +

+    -

+    -

+    -

+    -

+    -

GFP 1-275/GFP

B

GFP

1-275/GFP

1-482/GFP

972-1545/GFP

649-1658/GFP

GFP/1204-1658

DsRed-KRKTKISRKTR

GFP-KRKTKISRKTR

C

1608-1619 aa

1-116 aa

1-275 aa

1-482 aa

474-972 aa

649-1545 aa

649-1658 aa

1204-1658 aa

1546-1658 aa

DsRED - KRKTKISRKTR 1608 - 1619 aa

GFP - KRKTKISRKTR 1608 - 1619 aa

GFP/649-1545

GFP/1204-1658

649-1658/GFP

GFP/649-1545

GFP/1204-1658

GFP-KRKTKISRKTR

GFP/649-1545

GFP/1204-1658
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Svetlana A Didichenko and Marcus Thelen

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