PHD THESIS

NUCLEAR / NUCLEOLAR LOCALISATION AND TRANSPORT MECHANISM OF PHOSPHATIDYLINOSITOL 4-KINASE ISOFORM PI4K230

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2008

INTRODUCTION

Phosphoinositides account for only a tiny fraction of cellular phospholipids but are extremely important in the regulation of such diverse processes as signal transduction by hormones and neurotransmitters, ion channel activity, cytoskeletal reorganisation, vesicular trafficking and membrane fusion.

Phosphoinositides are derivatives of phosphatidylinositol (PtdIns) in which one or more of the OH groups at the 3-, 4-, 5-position of the inositol ring are phosphorylated in all possible combinations. Several kinases and phosphatases that interconvert phosphoinositides have been identified.

Phosphatidylinositol 4-kinases (PI4K) catalyze the production of PtdIns 4(P) from PtdIns, the first step in the formation of PtdIns (4,5)P_2 and PtdIns (3,4,5)P_3, two lipid products whose functions as regulatory molecules are best understood. PtdIns (4,5)P_2 is the main substrate of phospholipase C (PLC) enzymes, yielding inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), two important messenger in signalling. PtdIns (3,4,5)P_3 regulates a wide range of processes, such as cell metabolism and anti-apoptotic pathways via the serine/threonine kinase Akt, but also controls tyrosin kinases (Btk) and guanine exchange factors for small GTP-binding proteins. Beside these, phosphoinositides control a variety of cellular signalling and trafficking processes by recruiting regulatory proteins to cellular membranes through their protein modules (PH, ENTH, FYVE, PX, PHD, DHR) that can recognise specific isomers of phosphoinositide molecules.

The production of these signalling phosphoinositides relies upon the activity of their synthesizing enzymes, and PI4Ks have a pivotal role in this respect. In mammals, four different PI4K isoforms have been cloned with different subcellular distribution and enzymological properties: PI4K55α, PI4K55β, PI4K92, PI4K230. All of these forms are present simultaneously in several tissues, nevertheless PI4K230 shows the highest expression in the central nervous system. PI4K55α and β are involved in the production of the second messengers IP_3 and DAG at the plasma membrane, moreover in the endo- and exocytotic pathways in the cytoplasm. PI4K92 exhibits characteristic association with the outer surface of cisterns and vesicles of the Golgi and is the key enzyme for the regulation of Golgi function. PI4K230 is localized at the perikarya and small vesicules in close vicinity to synaptic membranes, cisterns of the rough endoplasmic reticulum and outer membranes of mitochondria. These observations are consistent with the involvement of all isoforms at several sites of vesicular trafficking.

PI4Ks have similar C-terminally located catalytic domains and very diverse N-terminally located regulatory domains. Amino acid sequence analysis revealed putative functional domains in the various isoforms of PI4Ks that could play roles in protein-protein and protein-lipid interactions. PI4K230 contains quite a variety of domains for protein-DNA interaction whereas PI4K92 and PI4K55α and β are much simpler in this respect. Nucleo-cytoplasmic traffic is suggested for all PI4K isoforms based on their putative nuclear localisation signals (NLS).

Phosphoinositides regulate diverse arrays of cytoplasmic processes, however phosphoinositides and enzymes responsible for their generation are also present in the nucleus of mammalian cells. Nuclear phosphoinositides are not only concentrated in the nuclear envelope but also located inside the nucleus forming a non-ionic detergent resistant fraction. The regulation of nuclear phospholipid metabolism is independent from that of the plasma membrane. Nuclear phosphoinositides nowadays receive increasing attention, because they play roles in the regulation of chromatin structure, transcription, cell proliferation, differentiation and apoptosis. Monitoring PtdIns (4,5)P_2 suggests the existence of several spatially and regulationally separated phosphoinositide cycles located in different subdomains of the nucleus. Although the formation of PI4P is a rate limiting step in the production of phosphoinositide derivatives, the nuclear occurrence of PI4-kinases is less known. PI4-kinases have only been detected in small restricted areas of the nucleus; for example, PI4K92 was associated with speckles and lamina-pore complexes, and PI4K55 was associated with the actin filament fraction. In contrast to predictions based on amino acid sequences (several NLSs and DNA binding motifs), there was no detectable nuclear localisation of PI4K230 which otherwise would be a potential candidate for this role.

Nucleocytoplasmic transport

Bidirectional nuclear transport of macromolecules takes place through large protein channels termed nuclear pore complex (NPC) that are embedded in the nuclear membrane. Ions, metabolites, and small proteins (less than ~40kDa) move through the NPC by passive diffusion, but the majority of larger molecules is transported using signal- and energy-dependent mechanisms. Most signal-dependent transport is mediated by nucleocytoplasmic shuttling receptors of the importin β (karyopherin) family. NLSs or nuclear export signals (NES) in proteins are recognized directly by transport receptors or indirectly via adaptor proteins that binds to the receptor. The most typical adaptor protein is importin α that after forming a complex with importin β directly binds classical NLSs (cNLS) of

proteins with high affinity. Cargo binding and release of importins are controlled by a RanGTP gradient across the nuclear envelope. Low levels of RanGTP in the cytoplasm allow the binding of importins to their cargoes, whereas high RanGTP levels in the nucleus induce their dissociation. RCC1, the guanine nucleotide exchange factor stimulates the conversion of RanGDP into RanGTP in the nucleus and RanGTPase activating protein (RanGAP1) together with Ran binding proteins (RanBP1 or RanBP2), stimulates RanGTP hydrolysis in the cytoplasm, while nuclear transport factor (NTF2) recirculates cytoplasmic RanGDP into the nucleus.

Whereas import of a protein into the nucleus is generally considered to be mediated by specific basic stretches named mono or bipartite cNLSs, it is becoming clear that efficient and well controlled import of proteins can also occur in the presence of non-classical NLS (ncNLS). In the case of nucleolar localisation, no obvious consensus sequence for the transport of proteins to the nucleolus is known but these nucleolar targeting signals (NTS) contain high proportion of basic residues and often resemble to NLSs .

AIMS

In spite of cumulating evidence for the distinct roles for PI4K230 in global cellular functions, there was no previous observation about the nuclear occurrence of PI4K230. Furthermore, not only putative NLS sequences suggest its nuclear localisation but a bZip transcription factor basic domain, leucine zippers and helix-loop-helix motifs. These motifs raise the possibility, that this protein might itself either function as a transcription factor within the nucleus or otherwise serve to modify gene transcription (for example, by competing with one or more nuclear transcription factors).

In our preliminary experiments, immunofluorescent detection of PI4K230 revealed a prominent staining in the nucleolus of neuronal cells. This observation deserves particular interest in light of the diverse functions of nuclear phosphoinositides. We have pursued the following questions:

- We have set out to verify the nuclear occurrence of native PI4K230 in neuronal tissue sections and in various cell lines, and to examine the subcellular localisation of expressed recombinant and deletion mutant forms. RNA interference was planned to verify the specificity of immunostaining.

- As a first step toward identifying the potential biological significance of nucleolar PI4K230, we undertook to determine the molecular mechanism by which this protein enters the nucleus and to identify the component(s) of the nucleolus with which PI4K230 might interact.

- A mono and a bipartite nuclear localisation signals are suggested in the domain structure of PI4K230, however, there is no evidence that these basic stretches really contribute to the nucleo-cytoplasmic shuttling of the enzyme. This led us to analyse the possible role of the putative NLS sequences in targeting PI4K230 to the nucleus.

-Similarly to the characterization of NLS sequences, we also wanted to identify NTS sequences responsible for directing PI4K230 protein to the nucleolus.

MATERIALS AND METHODS

Immunohistochemistry of rat brain and spinal cord

Adult Wistar rats were transcardially perfused with Ringer solution followed by a fixative containing 4% (w/v) freshly depolymerized paraformaldehyde (PFA) in 0.1 M PBS for 30 min. at RT. Brain and spinal cord were dissected out, cut into frontal blocks.

For native cryosections rats were decapitated, brain and spinal cord were quickly dissected out, cut into blocks and frozen immediately. 20 µm quick frozen cryosections were either treated with 100% ethanol at -20°C or with 4% (w/v) freshly depolymerized PFA. Some sections were pretreated with PBS for 10 min. before PFA fixation.

Fixed sections were washed with PBS and blocked (10% normal goat serum in TX-TPBS: 0.5% w/v Triton X-100, 10 mM Tris.HCl in PBS, pH 7.4), then they were incubated with anti-PI4K230 or anti-PI4K92 antibodies. After a PBS wash, Alexa Fluor 488 labelled secondary antibodies were used for visualisation. In controls, the sections were incubated with non-specific chicken IgY preparation, or only with the secondary antibody.

Epitope retrieval with citrate buffer

After PFA fixation, slides were immersed in 10 mM citrate buffer, pH 6.0 and heated until boiling. After removal, they were washed with cold PBS, blocked and processed as described above.

Cell culture and immunocytochemistry

HCN-1A (primary human cortical neuronal cells), B50 (rat neuroblastoma), COS-7 (African green monkey kidney) and HeLa (human cervical adenocarcinoma) cells were grown in DMEM supplemented with 10% FCS and antibiotics. Cells were subcultured using trypsin-EDTA detachment and seeded for immunocytochemistry on 12 mm glass coverslips.

The solutions for fixation, blocking, permeabilization and immunostaining of the cells were almost the same as described under immunohistochemistry. Incubations with all primary and secondary antibodies were performed in TX-TPBS containing 5% normal serum, and lasted for 1 h. Primary antibodies used were: anti-PI4K230 against aa 873-1145m, aa 1201-1220, aa 1361-1378, and aa 1566-1863; anti-PI4K92; anti-PI4K55α; anti-nucleolin; anti-fibrillarin; anti-hemagglutinin. Appropriate secondary antibodies were AlexaFluor labelled. In some experiments propidium idodide was applied at 1 µg/ml for 5 min to stain the nucleus.

Treatment of B50 cells with Triton X-100, dezoxyribonuclease I and ribonuclease A

B50 cells were treated with TX-TPBS before fixation in the presence of proteinase inhibitors (aprotinin, leupeptin, chymostatin and TPCK) for 15 min at RT. Digestion with DNase I (17 μg/ml in 10 mM Tris.HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and proteinase inhibitors, pH 7.5,) or RNase A (70 μg/ml in 50 mM Tris.HCl, 5 mM EDTA and proteinase inhibitors, pH 7.5) was performed for 20 min following permeabilization with TX-TPBS. Cells were then PBS washed, fixed with ethanol, blocked and stained as above.

siRNA treatment of COS-7 cells and RT-PCR

COS-7 cells at 30-40% confluence were transfected with 0.2 μM double–stranded siRNA corresponding to nucleotides 1072-1092 of PI4K230 (Qiagen) using oligofectamine. After 6 h the medium was changed to DMEM containing 10% FCS. Transfection with siRNA was repeated 24 h later. Cells were studied on the third day by immunocytochemistry.

The mRNA level of PI4K230 and GAPDH was determined by RT-PCR analysis from both siRNA-treated and control COS-7 cells. 24 h after siRNA transfection total RNA was isolated. The assay mixture for reverse transcriptase reaction contained 2 μg RNA, 1 μg random hexamer, 0.5 mM dNTP, 1 U/μl RNasin, 200 units M-MLV RT in 1x RT Buffer. The primers used in the PCR reaction for PI4K230 were: *5'-AACATGGAA-GAGTCTCTCCTCTC-3'* and *5'-CCATCACACTGGCTACAATG-3'* GAPDH was used as control. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide.

Expression of PI4K230 and its deletion mutant forms in COS-7 cells.

cDNAs of wild type and deletion mutant (Δ130, Δ97, Δ68, Δ56) PI4K230 cloned into pHM6 or pEGFP plasmids, respectively, were kindly provided by L. M. G. Heilmeyer (Ruhr Universität, Bochum). COS-7 cells at 60-70% confluency were transfected with expression constructs by Fugene 6 transfection reagens. After one day (PI4KΔ67 and PI4KΔ56) or two days (PI4K230, PI4KΔ130, PI4KΔ56) of expression cells were fixed with ethanol and localisation of expressed proteins was detected through indirect immunofluorescence of the labelled HA-tag on PI4K230 or direct fluorescence of the GFP-tag on deletion mutants.

Cloning and expression of PI4K230 in Sf9 insect cells

Sf9 cells (Spodoptera frugiperda) were grown in monolayer at 27 ˚C in SF-900 II SFM medium containing 10 μg/ml gemtamycin.

The complete coding region of PI4K230 was subcloned from pHM6-PI4K230 into a StuI and NotI digested pFastBAC HTb vector behind a His_6-tag coding region. This construct was transposed into a bacmid DNA as described by the manufacturer (Gibco). After transfection of Sf9 cells with bacmid DNA (as per the manufacturer's instruction) recombinant baculovirus particles were isolated from the medium after 72 h. Sf9 cells at a density of $2x10^5$ cells / cm^2 were infected with the amplified virus particles at a multiplicity of 5 and harvested 96 h. later. Cells were lysed in ice cold lysis buffer (for activity measurement: 20 mM KH_2PO_4 pH 7.0, 150 mM NaCl, 1% (v/v) TX-100, 0.5 mM EGTA, 1 mM PMSF, 1 µg/ml pepstatin, 10 µg/ml aprotinin, 10 µg/ml leupeptin and for import assay: 60 µg/ml digitonin in import buffer containing the protease inhibitors listed in import assay) by vortexing for 1 min and sonication.

Measurement of PI4K activity

The reaction mixture contained: 0.83 mg/ml PtdIns; 5 mM [γ-^{32}P] ATP (400-800 Bq/nmol); 27 mM $MgCl_2$; 116 mM KCl; 116 mM Hepes / KOH; 1mM EDTA; 1 mM EGTA; 1 mM DTE; 0.4% (v/v) Triton X-100 (pH 7.5). It was incubated at 25 ˚C for 15-30 min. The radioactive phosphate incorporated into PtdIns was measured after clorophorm-methanol extraction.

Bacterial protein expression and purification

Expression constructs encoding RanGAP, importin α1 and importin β were kindly provided by Dirk Görlich (University of Heidelberg, Germany). Plasmids for expressing importin α3 and importin α5 were generous gift of Riku Fagerlund (National Public Health Institute, Helsinki) while the Ran coding construct was kindly provided by János Szabad (University of Szeged).

Proteins	**Vectors**	**Expr. strains**	**Purification**
Ran	*pQE-11*	*BLR*	*Ni-agarose*
RanGAP	*pQE-60*	*M15*	*Ni-agarose*
importin α1	*pQE-70*	*M15*	*Ni-agarosez*
importin α3	*pQE-60 (poli-His changed to GST-)*	*BL21*	*GSH-sepharose*
importin α5	*pQE-60 (poli-His changed to GST-)*	*BL21*	*GSH-sepharose*
importin β	*pQE-60*	*M15*	*Ni-agarose*

Conjugation of synthesized NLS peptides to fluorescent labelled BSA and trypsin inhibitor

To analyse the possible roles of the monopartite, bipartite and nonclassical NLS sequences of PI4K230 in targeting proteins to the nucleus and nucleolus, we had the following wild type and mutant peptides synthesized for coupling to Alexa-488 labelled BSA and trypsin inhibitor:

Name	Amino acid sequence of synthetic peptides
NLS1	Cys-^{916}NFNHIHKRIRRVADKYLSG^{934}
NLS1-mut	Cys-^{916}NFNHIHAAIAAVADKYLSG^{934}
NLS2	Cys-^{1414}SKKTNRGSQLHKYYMKRRTL^{1433}
NLS2-mut	Cys-^{1414}SAATNRGSQLHKYYMMGMTL^{1433}
NLS2/1	Cys-^{1414}SKKTNRGSQLHKY^{1426}
NLS2/2	Cys-^{1427}YMKRRTL^{1433}
NLS-3	Cys-^{934}GLVDKFPHLLWSGTVLK^{950}

Trypsin inhibitor was reacted with AlexaFluor488-succinimidyl ester according to the manufacturer's instructions and washed with PBS using Centriprep 10Y column. The dye/protein ratio of Alexa488 labeled trypsin inhibitor was 3-4 mol/mol. The labeled Alexa488trypsin inhibitor (Alexa488trp.inh.) and Alexa488BSA (Invitrogen) was reacted with 1 mg sulfoSMCC in 1 ml 50 mM K-phosphate pH 7.2 for 1 hour at RT. SulfoSMCC was removed by four repeated dilutions (1:20) with PBS and concentration in Centriprep 10Y. Then the maleimide derivative was diluted with distilled water (bubbled with N_2), chilled to 4ºC and mixed with 10-fold molar excess of appropriate peptide. After stirring overnight at 4ºC, free maleimide was blocked with 3 mg L-Cys in 50 mM Tris-HCl pH 7.2 for 1 hour, RT. The preparation was concentrated and washed with transport buffer by repeated dilutions and concentrations. The peptide/protein ratio was determined by SDS-PAGE from the shift of the modified protein, giving an average ratio of 3-5 for BSA derivatives and 1-3 for modified trypsin inhibitor.

Cloning, expression, purification and labelling of epxr.NLS2

Upon PCR amplification of the 3498-5001 bp fragment of the PI4K230 cDNA EcoRI and XhoI restriction sites were created at the 5' and 3' ends respectively with primers:
5'-ATATGAATTCGAGCATGGCATGGAGACGG-3', and

5'-CCGAGGAACAGGCCGGGAGCTCAATT-3'. The product was inserted into a pET-28a plasmid to generate the pET28-NLS2 expression construct. The encoded 501 aa. polypeptide (comprising aa 1166-1667 of PI4K230) contains the bipartite NLS of the enzyme in its broad molecular environment.

The protein was expressed in BL21(DE3) E. coli, induced by 0,4 mM IPTG for 4 h at 28 °C. 50 mM Tris.HCl, H: 7.5, 100 mM NaCl, 5 mM Mg-acetate, 5% (v/v) glycerol and protease inhibitors were used to lyse sonicated cells. The same lysis buffer with 2-7 M urea was used to dissolve the expressed protein from precipitation after centrifuging the lysate. For purification, SP-sepharose chromatography was used and the eluted NLS2 protein was labelled using sulfhydryl labelling with Alexa488-maleimide according to the manufacturer's instructions.

Site-directed mutagenesis of basic amino acids critical in nuclear transport of expr.NLS2

Mutagenesis of the pET28-NLS2 vector was carried out by PCR utilizing the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. The sense primer for the Lys1415Ala and Lys1416Ala changes was:
5'-CAAGAAATCAGGCATGTCTGCGGCAACCAACCGGGGCTCCC-3'
and for the Lys1429Met, Arg1430Gly and Arg1431Met changes:
5'-GCACAAATACTACATGATGGGCATGACGCTGCTGCTGTCCC-3';
antisense primers were complementary, altered codons are underlined. The presence of the desired mutations was verified by DNA sequencing.

The mutated protein was expressed, purified and labelled similarly to expr.NLS2 (named as: expr.NLS2mut-Alexa488).

Nuclear import assay

To analyse the possible role of the predicted monopartite, bipartite and nonclassical NLS sequences of PI4K230 in targeting to the nucleolus, we used selectively permeabilized HeLa cells for the transport assay. HeLa cells were seeded on coverslips, rinsed with PBS and import buffer (20 mM HEPES / KOH, pH 7.3, 110 mM K-acetate, 5 mM Na-acetate, 2 mM Mg-acetate, 1 mM EGTA, 2 mM DTT), permeabilized for 7 min with import buffer containing 60 µg/ml digitonin, and washed 3x with import buffer. Sf9 lysate containing PI4K230, expr.NLS2-Alexa488 (700 nM) or NLS peptides conjugated to Alexa488BSA or Alex488.trypsin inhibitor (0.1 mg/ml each) were used for 15 min as import cargo. These substrates were dissolved either in a mixture containing reticulocyte lysate (150 µg/ml), ATP regenerating system (1 mM ATP, 0.5 mM GTP, 10 mM creatin-

phosphate, 50 µg/ml creatin-phosphokinase) and protease inhibitors (aprotinin, leupeptin, pepstatin 1 µg/ml each) in import buffer, or in a mixture containing ATP regenerating system, Ran mix, (3 µM Ran-GDP, 150 nM RanGAP, 300 nM NTF2), importins (α1, α3, α5, β, 1µM each) and protease inhibitors in import buffer. Cells were then washed, fixed with abs. ethanol (-20 ˚C, 10 min), labelled fluorescently to visualize the nucleus, and the nucleolus, and examined by confocal microscopy.

The dependence of transport on guanine nucleotide-binding proteins was tested using the nonhydrolisable GTP analog GTPγS (0,2 mM). Specific transport through nuclear pore complexes was inhibited by the lectin wheat germ agglutinin (WGA) that was applied at 20 µg/ml concentration for 5 minutes before as well as during the import assay. As positive and negative controls, we used the critical NLS of SV40 large T antigen fused to rhodamine labelled bovine serum albumin, and Alexa488BSA (both at 700 nM).

Determination of protein-protein interaction by dot blot overlay experiments

1.5 µg of NLS-Alexa488BSA derivatives and the expr.NLS2-Alexa488 were spotted onto nitrocellulose strips and blocked with 5% (w/v) BSA and 20 µg/ml leupeptin in transport buffer for 4h. Hybridization was performed with 1 µM of importin(s) in import buffer containing 1% BSA and 20 µg/ml leupeptin for 16 hours at 4ºC. Precomplexation of importin α and β (molar ratio of 1:1) was carried out for 15-30 min at RT. After PBS washing the membranes were incubated with anti-GST (20 ng/ml) and peroxidase-labeled secondary antibodies (2 ng/ml) dissolved in PBS containing 1% BSA or HRP-anti-poly-His (5 ng/ml) in the same buffer. Immunoreactions were detected by ECL on X-ray film.

Western blotting

Samples were boiled in Laemmli's buffer for 10 min, subjected to SDS-PAGE, electrotransferred to PVDF membrane and blocked with 1% normal serum and 5% skim milk in PBS / 0.1% Tween-20. The four primary antibodies against PI4K230 were the same as for immunohistochemistry. Binding was detected by enhanced chemiluminescence using HRP-conjugated secondary antibodies.

Confocal microscopy

For confocal laser scanning microscopy, a Zeiss LSM 510 system with a Plan-Apochromat 63×/1.4 NA immersion objective was used. Fluorophores were excited with Ar ion (488 nm) or HeNe (543 nm or 633

nm) lasers and detected through a 505-550 nm, 560-615 nm or 585-615 nm emission filters or 650 nm longpass filter. Pinholes were set to obtain 1 μm optical sections. 512×512 pixel images were taken at 12 bit resolution and 2× line-averaging.

RESULTS AND DISCUSSION

Specific detection of PI4K230 in the nucleolus of ethanol fixed cells

The antibody against aa 873-1145 PI4K230 (termed anti-N) detected a prominent signal in the nucleolus of ethanol fixed primary neuronal HCN-1A cells in addition to the strong immunoreactivity in the cytoplasm. In contrast, the nucleolar staining was absent from PFA fixed cells. We also performed the immunofluorescent detection of PI4K230 in spinal cord sections obtained from PFA perfused rats and in ethanol fixed cryosections. PI4K230 immunofluorescence could only be observed in the cytoplasm of spinal cord neurons of PFA perfused rats and of quickfrozen sections that were post-fixed in PFA. On the other hand, ethanol fixed cryosections reveal a prominent signal in the nucleolus and a faint to moderate staining of the nucleus, in addition to the strong immunoreactivity in the cytoplasm. In ethanol fixed controls, only a slight background staining was observed either when the first antibody was omitted or replaced with nonspecific chicken IgY. As opposed to PI4K230, immunofluorescent staining of PI4K92 in ethanol fixed cryosections of spinal cord revealed no nucleolar signal.

The difference in staining pattern of PI4K230 between PFA and ethanol fixed tissues was observed in various brain areas. The sections obtained from the parietal cortex, hippocampus or cerebellar cortex all show a characteristic nucleolar staining only in ethanol fixed cryosections but not in sections from PFA perfused rats.

To rule out the possibility of non-specific staining or cross-reactivity with some other peptide sequence, we tested several antibodies raised against different sequences of PI4K230. Results on ethanol and PFA-fixed B50 cells identical to those with the anti-N antibody were obtained with antibodies against aa 1361-1378, aa 1201-1220 and aa 1566-1863 of PI4K230 sequences using Alexa Fluor 488 conjugated specific secondary antibodies. (Specificity of all antibodies was confirmed on immunoblot of PI4K230 expressed in Sf9 cells.) Co-localisation of the prominent PI4K230 signal with nucleolin in ethanol fixed cells indicated its nucleolar localisation in all cases. In formaldehyde fixed cells, only nucleolin labeling was seen without any well-defined co-staining with PI4K230; however, PI4K230 was well detected in the cytoplasm and to some extent in the nucleus of these cells, indicating that the masking effect of PFA fixation is restricted only to the nucleolar PI4K230. Methanol-acetic acid or acetone fixation gave a similar picture to that obtained with ethanol, suggesting that chemical reactions of formaldehyde can be responsible for masking the immunoreaction of PI4K230 in the nucleolus.

The exact localisation of PI4K230 inside the nucleolus was revealed on ethanol fixed HeLa cells by co-labelling the enzyme and the nuclear marker proteins nucleolin and fibrillarin. Based on colocalisation studies, PI4K230 exhibits a well defined occurrence in the dens fibrillar component but a complete lack of appearance in the dense fibrillar center.

We also tested the difference between ethanol and PFA fixation on the staining pattern of PI4K92 and PI4K55α in B50 cells. These experiments showed that the fixation method does not cause major differences in the staining pattern of these PI4K isoforms. PI4K92 shows an intensive labelling of the Golgi and a slight to moderate diffuse granular staining in the cytoplasm. In the nucleus, only faint scattered granular staining can be observed without any concentration in the nucleolus. PI4K55α displays a moderate granular cytoplasmic and a more intense nuclear staining with both fixation methods, nevertheless, a distinct, prominent nucleolar signal is missing.

Testing the direct effect of PFA on the immunoreactivity of PI4K230

Lysates of Sf9 cells containing PI4K230 were treated with PFA at increasing concentrations (0-1%) for 10 min and separated by SDS-PAGE. Immunoblotting using anti-N as primary antibody indicated, that PI4K230 remains detectable despite PFA treatment. The same result was obtained when PI4K230 was FPA-fixed on the PVDF membrane. These observations suggest that epitope masking by PFA is not caused by intramolecular but intermolecular crosslinks between PI4K230 and nucleolar interaction partners.

Unmasking the nucleolar staining of PI4K230 in PFA fixed cells

Since the nucleolar staining of PI4K230 was seen only without formaldehyde fixation, we reasoned that during PFA fixation the immunoreactive epitopes had become masked. Indeed, the masked epitopes in PFA fixed B50 cells could be retrieved by heating PFA fixed cells in citrate buffer, a method often used for retrieval of epitopes buried by PFA fixation. Interestingly, the masking effect of PFA could be prevented if the quick frozen sections were pre-washed with PBS before PFA fixation, probably because such a time period is long enough to remove or digest the cross-linkable partners.

Effect of siRNA treatment on the nucleolar staining of PI4K230

To further verify the validity of the nucleolar immunoreaction of PI4K230, we tested the effect of siRNA treatment designed to interfere with the expression of PI4K230. Repeated treatment of COS-7 cells with siRNA

caused the disappearance of the vast majority of PI4K230 immunoreactivity in ethanol fixed cells, including the nucleolar staining, leaving only a faint to moderate, diffuse nuclear immunoreactivity by the third day. The efficiency of the knock-down on PI4K230 mRNA levels was assessed after 24 h of treatment since longer treatment was toxic causing many cells to detach. At 24 h the effect of siRNA was already apparent in the cell population resulting in a decrease in the level of PI4K230 specific mRNA, while the amount of mRNA specific for the control GAPDH did not change.

Effect of Triton X-100 on the subcellular distribution of PI4K230

Because some phosphoinositide kinases in the nucleus were demonstrated in a detergent-insoluble protein-lipid complex, we tested the effect of TX-100 on the subcellular distribution of PI4K230. A 5-minute TX-100 treatment of B50 cells before fixation resulted in a decrease of cytoplasmic and a moderate increase of nuclear immunostaining. Parallel labelling of nucleolin gave a diminished and somewhat scattered signal indicating the overall destructive effect of the detergent on the nucleoli. Quantitative image analysis of PI4K230 density in confocal sections of cells treated with the detergent for various times (in the range of 5 – 60 min) revealed that a large proportion of the enzyme is quickly, within 5 min, removed by Triton X-100, and the source of this fraction is primarily the cytoplasm. By this time, there appears a slight increase in the nucleus, and decrease in the nucleolus. After longer incubations, the density in the cytoplasm reaches a steady state, while the nucleolar fraction decreases at a low but constant rate. Nonetheless, nucleolar PI4K230 immunoreactivity remains prominent throughout. These observations together suggest that most PI4K230 binds to the nucleolar compartment in a Triton X-100 resistant form.

Effect of DNase and RNase pre-treatment on the nucleolar localization of PI4K230.

To shed light on the component(s) that could bind to PI4K230 in the nucleolus, we tested the effect of DNase and RNase treatment on PI4K230 immunostaining. Though disruption of the nuclear envelope by TX-100 did not influence the prominent nucleolar staining of PI4K230 in permeabilized B50 cells, DNase I or RNase A treatment of TX-100 permeabilized cells caused the loss of nucleolar PI4K230 signal. Mobilizing PI4K230 from the nucleolus with DNase or RNase suggests its association in a complex with nucleic acids.

Subcellular localisation of expressed PI4K230 and its deletion mutant forms

After 2 days of expression, neither the full length PI4K230 nor its deletion mutants were accumulated in the nucleolus, while endogenous PI4K230 exhibited nucleolar localisation in transfected COS-7 cells. Otherwise, in some cases we observed nucleolar translocation of PI4KΔ130 and nuclear accumulation of expressed PI4K230 when the Crm1 export system was inhibited by Leptomycin B treatment.

Nuclear import of expressed PI4K230 in permeabilized HeLa cells

As a first experiment, we found that PI4K230 expressed in Sf9 cells exhibited a well defined nuclear transport in digitonin permeabilized HeLa cells supplemented with reticulocyte lysate and energy regenerating system. WGA, a lectin that inhibits receptor-mediated transport, prevented nuclear import of PI4K230 in this system. As a control, classical SV40 T-antigen NLS fused to Alexa488BSA without and in the presence of WGA was tested and was found to be transported similarly to exogenous PI4K230, indicating the reliability of the employed procedure.

The monopartite NLS (NLS1) of PI4K230 carries nuclear targeting information

The amino acid sequence of PI4K230 contains two hypothetical classical nuclear localisation signal motifs, a mono- and a bipartite, designated NLS1 and NLS2, respectively. Another sequence of PI4K230 is highly homologous to the non classical NLS of phospholipid scramblase 1 (termed NLS3). To test whether these regions have NLS activity, peptides corresponding to these amino acid sequences were synthesized and conjugated to Alexa488BSA to test in a nuclear import assay. To rule out unspecific binding of cargos peptides where basic amino acids critical in the transport were also synthesized (termed NLS1mut and NLS2mut).

Diffuse nuclear accumulation of NLS1-Alexa488BSA but not NLS2-Alexa488BSA and NLS3 Alexa488BSA was detected when nuclear import assay was carried out with reticulocyte lysate. Import of NLS1-Alexa488BSA was reduced by the signal-dependent nuclear transport inhibitor WGA and inhibited when the same reaction was performed in the presence of GTPγS. NLS1mut- and NLS2mut-Alexa488BSA was also not transported. Interestingly, despite the observation that NLS2-Alexa488BSA does not show nuclear transport, its perinuclear accumulation suggests the ability of NLS2 binding to nuclear transport factors.

Nuclear import of NLS1 is mediated by the importin α1/β and α3/β heterodimers

Next we tested the participation of several importins in the transport of NLS derivatives. In place of the reticulocyte lysate, the import mix was supplemented with recombinant transport factors, Ran mix and NTF2. Nuclear accumulation of NLS1-Alexa488BSA was mediated by heterodimer of importins α1 and β or importins α3 and β, while neither single importins alone nor the importin α5/β complex could mediate nuclear import.

In vitro binding of NLS peptides to importins is coherent with their transport properties

Direct binding assay on dot-blot was carried out to assess recognition of NLSs by single importins and importin α/β complexes. Results support our previous observations: NLS1-Alexa488BSA is able to associate exclusively with importin α1/β and importin α3/β complexes but not with isolated importins, in contrast to the NLS1mut-Alexa488BSA that exhibits complete lack of binding similarly to NLS2mut-Alexa488BSA. Very weak or no association of NLS1 and NLS2 derivatives with importin α5 could be observed, even in the presence of importin β. NLS3-Alexa488BSA showed practically no association with importins α1, α3 or β, employed either alone or in the form of α/β complexes, similarly to Alexa488BSA which was used as negative control. Surprisingly, NLS2-Alexa488BSA showed preferential binding to importin α1/β and importin α3/β complexes and to importin β, which is coherent with its accumulation at the nuclear membrane but leaves the lack of its import unexplained.

Taken together, the observations suggest that nuclear import of PI4K230 could occur in a receptor dependent process mediated by the classical importin α1/β and importin α3/β pathway through a direct interaction with NLS1.

PI4K230(1166-1667) is effectively transported into the nucleolus

As noted above, synthesized NLS2-Alexa488BSA was not imported in to HeLa nuclei but bound to importins. Thus we also expressed NLS2 as a recombinant protein comprising aa 1166-1667 of PI4K230. Its 57 kDa molecular weight exceeds the diffusion limit of NPC. Alexa488 labeled expressed NLS2 showed not only nuclear transport, but also nucleolar accumulation. The import process was inhibited by WGA and GTPγS and mediated specifically by importin α1/β and importin α3/β but not by other combinations, coherent with dot-blot results. The mutated expressed NLS2 did not show nuclear transport and did not bind to these importins.

Consequently, this putative bipartite NLS2 of PI4K230 could act as functional NLS when placed in its broader molecular environment, which emphasizes the importance of flanking amino acids that could influence the ability of the signal sequence to mediate import. Interestingly, a synthetic peptide comprising more of the flanking regions of NLS2 did not show the same α/β mediated transport as expr.NLS2-Alexa488.

NLS2 carries nucleolar targeting information

Previous results led us to identify the sequences responsible for directing expr.NLS2-Alexa488 to the nucleolus. Although no consensus nucleolar targeting signals (NTS) are defined, a high proportion of basic residues similar to those in classical NLSs are expected in them. A part of the amino acid sequence of PI4K230, largely constituting the second basic stretch of the bipartite NLS2, shows high homolgy with the NTS of human angiogenin. Thus we have investigated the nucleolar directing capability of 3 synthetic peptides corresponding to the first basic amino acid cluster of NLS2 (named NLS2/1), the angiogenin NTS homologue fragment (named NLS2/2) and the full length NLS2. The size of these synthetic peptides conjugated to Alexa488-trypsin inhibitor (Alexa488trp.inh) is smaller than the molecular weight cut-off limit of the nuclear pore complex. Consequently, these molecules in nuclear import studies are able to diffuse freely between the cytoplasm and nucleoplasm and accumulation can only occur through binding to nuclear/nucleolar components.

Alexa488trp.inh alone as control showed no signal in the nucleus of permeabilized HeLa cells. NLS1-Alexa488trp.inh was almost completely excluded from the nucleus / nucleolus, suggesting that the NLS1 fragment of PI4K230 is not responsible for nucleolar direction and retention. On top a a light fluorescence throughout nucleus, a more prominent nucleolar signal of NLS2/1-Alexa488trp.inh and a weaker one of NLS2/2-Alexa488trp.inh was seen. The complete NLS2-Alexa488trp.inh was enriched in nucleoli even more than its fragments, which again emphasizes the importance of the actual molecular environment of NLS sequences.

NLS2/1, NLS2/2, and full length NLS2 when fused to NLS1-Alexa488-BSA also showed nucleolar accumulation at similar proportions as the tryp.inh linked counterparts. The nucleolar directing force of full length NLS2 is best characterized by the import of NLS2-NLS1-Alexa488BSA where all of imported molecules are accumulated in the nucleolus. The results indicate that NLS1 sequence confers exclusively nuclear directing information clearly distinct from NLS2 which is able to mediate nucleolar and – with its molecular niche – nuclear import as well.

CONCLUSIONS

- The presence of PI4K230 in the nucleoli of ethanol fixed neuronal and non-neuronal cells with indirect immunofluorescence using antibodies to four of its distinct epitopes. Based on our colocalisation results, nucleolar PI4K230 exhibits prominent staining in dense fibrillar components.
- PFA treatment had no effect on immunoreactivity of PI4K230 in cell lysate or immunoblot but masked reversibly the immunoreactive epitopes of PI4IK230 in the nucleolus by crosslinking PI4K230 with tightly associated nucleolar components.
- In experiments with siRNA interfering with the expression of PI4K230, elimination of PI4K230 immunoreactivity from the cytoplasm and nucleoli was observed, while the remaining PI4K230 accumulated in the nucleus.
- The PI4K230 exists in detergent-resistant proteolipid complexes of the nucleolus interacting with nucleolar DNA and/or RNA, either directly or through associated nucleic acid-bound constituents.
- Though recombinant PI4K230 expressed in COS-7 cells does not accumulate in the nucleolus even after 2 days but in transport assays PI4K230 expressed in Sf9 cells enriches in the nucleoplasm of digitonin permeabilized HeLa cells.
- Nuclear import directed by the monopartite NLS (NLS1) of PI4K230 is a WGA-sensitive and energy-dependent process mediated by importin α1/β and importin α3/β complexes that interact directly with NLS1.
- An expressed 506 amino aid fragment of PI4K230 containing its bipartite NLS is effectively transported to the nucleolus with importin α1/β and importin α3/β heterodimers in a WGA-sensitive and energy-dependent manner. Although the bipartite NLS sequence (NLS2) itself linked to BSA does not translocate to the nucleus, but when linked to trypsin inhibitor that small enough to pass freely the NPC it directs the cargo to the nucleolus. In this capacity, the complete NLS2 is more potent than either of its two fragments.

This thesis is based on the following publications:

Heilmeyer L. M. G. jr., Vereb G. Jr., Vereb G., **Kakuk A.,** Szivák I. (2003): Mammalian phosphatidylinositol 4-kinases. IUBMB Life 55.: 59-65. (IF: 2.3)

Kakuk A., Friedländer E., Vereb G. Jr., Kása A., Balla A., Balla T., Heilmeyer L. M. G. Jr., Gergely P., Vereb G. (2006) Nucleolar localization of phosphatidylinositol 4-kinasae PI4K230 in various mammalian cells. Cytometry A 69A: 1174-1183 (IF: 3.3)

Kakuk A., Friedländer E., Vereb G. Jr., Lisboa D., Bagossi P., Tóth G., Gergely P., Vereb G. Nuclear and nucleolar localization signals and their targeting function in phosphatidylinositol 4-kinase PI4K230. Experimental Cell Research (accepted) (IF_{2006}: 3.7)

Presentations, posters

Gülkan H., Suer S., **Kakuk A.**, Szivák I. (2001): Phosphatidylinositol 4-kinases. International Symposium on Structures and Mechanisms in Biological Signal and Energy Transduction. Dabringhausen, Germany. 25.

Kakuk A., Vereb Gy., Petrasch Parwez E., Heilmeyer L. M. G., Gergely P. (2001): Immunofluorescent detection of PI4K230 phosphatidylinositol 4-kinase in the nucleolus of neuronal cells. (in Hungarian) IX. Cell and Developmental Biology Days, Debrecen, Hungary 108.

Friedlander E., **Kakuk A.** (2002): Localisation of phosphatidylinositol 4-kinase isoform PI4K230 in neuronal cells. (in Hungarian) IX. Scientific Student Conference, Marosvásárhely, Románia. 32.

Kakuk A., Vereb Gy., Gergely P., Friedlander E., ifj. Vereb Gy. (2003): Examinaton of subcellular localisation of expressed phosphatidylinositol 4-kinase isoform PI4K230 and its deletion mutant forms in COS-7 cells. (in Hungarian) XI. Cell and Developmental Biology Days Siófok, Hungary 10.

Kakuk A., Vereb Gy., Gergely P., Zsebik B., ifj. Vereb Gy. (2003): Subcellular localisation of phosphatidylinositol 4-kinase isoform PI4K230 and its deletion mutant forms. (in Hungarian), 8th Meeting of Molecular Biology Section of Hungarian Biochemical Society Tihany, Hungary 42.

Kakuk A., Zsebik B., ifj. Vereb Gy., Gergely P., Vereb Gy. (2003): Examination of nuclear translocation of phosphatidylinositol 4-kinase

isoform PI4K230. (in Hungarian) II. Cell Signalling Congress of Hungarian Biochemical Society, Hőgyész, Hungary 41.

Kakuk A. (2003): Examinaton of subcellular localisation of expressed phosphatidylinositol 4-kinase isoform PI4K230 and its deletion mutant forms in COS-7 cells. (in Hungarian) Scientific Student Conference, Debrecen, Hungary 78.

Kakuk A., Zsebik B., ifj. Vereb Gy., Gergely P., Vereb Gy. (2004): The nuclear transport of phosphatidylinositol 4-kinase PI4K230. (in Hungarian) 9th Meeting of Molecular Biology Section of Hungarian Biochemical Society, Sopron, Hungary 138.

Kakuk A. (2004): Examination of nuclear translocation of phosphatidylinositol 4-kinase isoform PI4K230. (in Hungarian) Scientific Student Conference, Debrecen, Hungary 89.

Kakuk A., Szivák I., Vereb G. jr., Heilmeyer L. M. G. jr., Vereb G. (2004): Nuclear transport of the phosphatidylinositol 4-kinase isoform PI4K230. International Meeting on the Topogenesis of Organellar Proteins, Bochum, Germany. 54.

Friedländer E., **Kakuk A**,. Vereb Gy. Jr., Heilmeyer L.M.G., Gergely P., Vereb Gy. (2006): The "PI4K230" phosphatidylinositol 4-kinase exhibits nucleolar localisation in various mammalian cells. V. Hungarian Cellanalytical Conference, Budapest 75.

Kakuk A., Friedländer E., Vereb Gy. Jr, Petrasch-Parwez E., Balla T., Heilmeyer L, Gergely P., Vereb Gy. (2006): Nucleolar localization of phosphatidylinositol 4-kinase PI4K230 in various mammalian cells. 31st FEBS Congress, Istanbul, Türkey 161.

Kakuk A., Kása A., Friedländer E., Vereb Gy. jr., Balla A., Balla T., Vereb Gy. (2006) Examination of nuclear occurrence of the phosphatidylinositol 4-kinase isoform „PI4K230" a. Meeting of Hungarian Biochemical Society Pécs, Hungary 52.