Mapping of functional domains of the lipid kinase phosphatidylinositol 4-kinase type III alpha involved in enzymatic activity and hepatitis C virus replication

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Abstract

The lipid kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα) is an ER-resident enzyme that synthesizes phosphatidylinositol 4-phosphate (PI4P). PI4KIIIα is an essential host factor for hepatitis C virus (HCV) replication. Interaction with HCV non-structural protein 5A (NS5A) leads to kinase activation and accumulation of PI4P at intracellular membranes. In this study, we investigated structural requirements of PI4KIIIα involved in HCV replication and enzymatic activity. Therefore, we analyzed PI4KIIIα mutants for subcellular localization, reconstitution of HCV replication in PI4KIIIα knockdown cell lines, PI4P induction in HCV-positive cells and lipid kinase activity in vitro. All mutants still interacted with NS5A and localized similarly as the full-length enzyme, suggesting multiple regions of PI4KIIIα involved in NS5A interaction and subcellular localization. Interestingly, the N-terminal 1152 amino acids were dispensable for HCV replication, PI4P induction and enzymatic function, whereas further N-terminal or C-terminal deletions were deleterious, thereby defining the minimal PI4KIIIα core enzyme to a size of ca. 108 kDa. Additional deletion of predicted functional motifs within the C-terminal half of PI4KIIIα were also detrimental for enzymatic activity and for the ability of PI4KIIIα to rescue HCV replication, with the exception of a proposed nuclear localization signal, suggesting that the entire C-terminal half of PI4KIIIα is involved in the formation of a minimal enzymatic core. This view was supported by structural modeling of the PI4KIIIα C-terminus suggesting a catalytic center formed by an N- and C-terminal lobe and an armadillo-fold motif, which is preceded by three distinct alpha-helical domains probably involved in regulation of enzymatic activity.

Importance

The lipid kinase phosphatidylinositol 4-phosphate kinase type IIIα (PI4KIIIα, PIK4CA, PI4KA) is of central importance for the cellular phosphatidylinositol metabolism and a key host cell factor
of hepatitis C virus replication. However, little is known so far about the structure of this 240 kDa protein and the functional importance of specific subdomains regarding lipid kinase activity and viral replication. This work focuses on the phenotypic analysis of distinct PI4KIIIα mutants in different biochemical and cell-based assays and develops a structural model of the C-terminal enzymatic core. The results shed light on the structural and functional requirements of enzymatic activity and the determinants required for HCV replication.
Introduction

Worldwide about 170 million people are chronically infected with hepatitis C virus (HCV). HCV is a positive-strand RNA virus and belongs to the family of Flaviviridae. The viral genome encompasses about 9.6 kb and encodes mainly for a polyprotein of about 3,000 amino acids, which is flanked by nontranslated regions. The polyprotein is cleaved into ten mature proteins by cellular and viral proteases: core, envelope glycoprotein 1 (E1) and E2, p7 and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (reviewed in (1,2)). The structural proteins core, E1 and E2 are major constituents of the viral particle and, together with p7 and NS2, are mainly involved in the assembly of infectious virions, while NS3 to NS5B are required for replication of the viral genome. NS3 exhibits helicase and NTPase activities in the C-terminal part and an N-terminal protease constitutively bound to its cofactor NS4A. NS4B is involved in inducing membrane alterations that are required for viral replication (reviewed in (1)). NS5A is a phosphoprotein critically involved in various host factor interactions and in regulation of viral replication. Two distinct NS5A phospho-isoforms have been described, termed p56 and p58, which have been associated with HCV RNA replication and assembly, respectively (reviewed in (3)). NS5B is the viral RNA-dependent RNA-polymerase.

Viral RNA replication takes place in vesicular membrane alterations designated the membranous web (4,5) (MW), which consists of double membrane vesicles (DMVs) and multi-membrane vesicles (MMVs) (6-8). Although the precise role of these membrane alterations in RNA replication is still elusive, the appearance of DMVs correlates best with the kinetics of RNA synthesis and recent reports suggest that these structures indeed harbor the HCV replication sites (8,9). The mechanisms behind MW biogenesis are still poorly understood, however, combined action of all non-structural proteins seems to be required for MW formation (8). Furthermore, recent studies pointed to a crucial role of the cellular lipid kinase phosphatidylinositol 4-kinase
type IIIα (PI4KIIIα), which has been identified to contribute to the morphology of the MW by interaction with NS5A (7,10,11).

PI4KIIIα (PIK4CA, PI4KA) is an ER-resident enzyme of about 240 kDa in size and converts phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI4P). PI4P plays a role as precursor for synthesis of other phosphatidylinositides, but is also believed to take part in intracellular trafficking by constituting membrane identity and several proteins have been identified that specifically bind to PI4P (reviewed in (12)). PI4KIIIα was initially discovered by Gehrmann et al. by screening of a human fetal brain cDNA library (13) and is highly expressed in heart, brain, placenta, skeletal muscle, kidney, spleen, thymus, prostate, ovaries, small intestine and colon, and at low levels in lung, liver, pancreas, testis and leukocytes (14). Interestingly, in cDNA libraries of various tissues and cell lines, an alternative splice variant of PI4KIIIα was discovered lacking the coding sequence for the N-terminal 1249 amino acids of the 240 kDa variant. This truncated variant was found to be catalytically inactive in vitro and did not support HCV replication, pointing to an important role of residues further upstream in enzymatic function (15), but questioning the general significance of this isoform.

PI4KIIIα belongs to the family of mammalian PI4Ks, which comprises two types with two isoforms each (PI4KIIα, PI4KIIβ, PI4KIIIα and PI4KIIIβ) differing in their subcellular localization and being responsible for the synthesis of distinct PI4P pools (reviewed in (16)). While the yeast orthologue of PI4KIIIα is located at the plasma membrane (17), mammalian PI4KIIIα mainly resides at the ER (18), but is also located to Golgi membranes (19) and to the nucleoli upon exogenous expression (20). Localization of PI4KIIIα also differs among cell types, since in neuronal cells and other cell types like COS-7 or B50 cells it is prominently found in the nuclei (21). The exact role of PI4KIIIα at the ER still is unknown, but it was demonstrated that
PI4KIIIα is mainly responsible for PI4P synthesis at the ER, the plasma membrane (22, 23) and partly at the Golgi compartment (24).

Type III PI4Ks are structurally related to phosphatidylinositol 3-kinases (PI3K), since the C-terminal catalytic domain and a helical lipid kinase unique domain (LKU) are both highly conserved and both lipid kinase families share a common sensitivity towards wortmannin (16, 25). Besides that, little is known about structural determinants of PI4KIIIα playing a role in enzymatic function. The N-terminus of PI4KIIIα has no homology to other known proteins and comprises proline-rich regions and a putative SH3-domain, as determined by structural predictions of different groups (reviewed in (14)). In the center of the protein, nuclear localization signals (NLS) and leucine zippers (LZ) as well as a helix-loop-helix domain (HLH) are predicted (13, 14), which role in enzymatic function is still unclear. Also, PI4KIIIα has a predicted pleckstrin-homology domain (PH) between the LKU and the C-terminal catalytic domain (13), which, however, is not recognized by most algorithms and it is questionable if this domain is functional (16).

PI4Ks have been found to be important for replication of a number of viruses (reviewed in (26)). An essential role of PI4KIIIα in particular for HCV RNA replication has been identified by various studies (7, 11, 27-32). While the precise role of PI4KIIIα in the HCV replication cycle has not been clarified so far, several studies have shed light on distinct phenotypes modulated by PI4KIIIα. Knockdown of PI4KIIIα causes a “clustered” distribution of HCV nonstructural proteins in immunofluorescence (IF) (28), which is reflected by an altered ultrastructure of the MW showing DMVs with reduced diameter and lacking MMVs (7). It was observed that PI4KIIIα is responsible for the induction of PI4P at intracellular membranes in presence of HCV in cell culture and in vivo, which might be important for MW biogenesis (7, 33). A recent study has shown that increased PI4P levels induced by HCV recruit oxysterol-binding protein (OSBP),
thereby shuttling cholesterol to the MW (34), which is a critical component of the DMVs (9).

This work mechanistically links enhanced PI4P levels induced by HCV to the changes observed in MW morphology (34). Still, the impact of OSBP knockdown on HCV RNA replication is limited compared to knockdown of PI4KIIIα, suggesting that additional mechanisms involving PI4KIIIα might contribute to HCV RNA synthesis. Recently, it was shown that HCV also causes depletion of PI4P in the plasma membrane, which argues for a general reorganization of PI4P pools in presence of the virus (24). PI4KIIIα directly interacts with NS5A and NS5B (7,10,33).

Interaction of PI4KIIIα and NS5A was shown to be crucial for HCV replication and the functional interaction site for PI4KIIIα on NS5A was mapped to seven amino acids in domain 1 of NS5A (10). Mutations in that region as well as knockdown of PI4KIIIα result in increased levels of NS5A p58, whereas overexpression of PI4KIIIα favors synthesis of p56 (10). Therefore, the modulation of NS5A phosphorylation by PI4KIIIα might furthermore contribute to the role of this lipid kinase in HCV replication. The region in PI4KIIIα responsible for NS5A binding has been mapped to the N-terminal 400-600 amino acids of the kinase by another study (35).

However, the functional relevance of this region for HCV replication and PI4KIIIα activity has not been elucidated so far.

In this study, we shed light on structural determinants of PI4KIIIα important for enzymatic activity and HCV replication by providing a detailed characterization of different PI4KIIIα mutants. Furthermore, we give novel insights into the organization of C-terminal domains using molecular homology modeling and elucidate their contribution to the role of PI4KIIIα in HCV replication.
Materials and Methods

Cell lines

The human hepatoma cell line Huh7-Lunet stably expressing T7 RNA polymerase under zeocin selection was used for generation of cell lines and transient expression of plasmids encoding for HCV proteins analyzed in immunofluorescence and immunoprecipitation assays (36). Huh7-Lunet T7 cells transduced with non-targeting shRNA or with shRNA targeting endogenous PI4KIIIα under puromycin selection were described previously as well as PI4KIIIα knockdown cells reconstituted with shRNA-resistant full-length HA-PI4KIIIα (10). For reconstitution of PI4KIIIα expression, Huh7-Lunet T7 cells with stable knockdown of endogenous PI4KIIIα were generated accordingly by transduction of PI4KIIIα knockdown cells with lentiviral constructs encoding for shRNA-resistant HA-PI4KIIIα mutants. PI4KIIIα variants for purification purposes were expressed in Huh7-Lunet harboring T7 RNA polymerase under blasticidin selection, transduced with shRNA targeting endogenous PI4KIIIα.

Primers and plasmid constructs

The lentiviral vector pWPI-BLR (36) was used for cloning of plasmids encoding for different HA-tagged PI4KIIIα mutants for the generation of stable cell lines under blasticidin selection. The gene encoding the ca. 240 kDa full-length PI4KIIIα isoform 2 was originally obtained from Kazusa DNA Research Institute, Chiba, Japan (product ID FXC00322, corresponding to GenBank accession number AB384703, numberings refer to this GenBank ID). Notably, this PI4KIIIα construct harbors an additional Valin residue at the C-terminus (position 2103; GenBank entry AB384703), which was not present in other common GenBank entries of PI4KIIIα (accession: NP_477352.3), but did not affect any function of the kinase (data not shown). Importantly, the PI4KIIIα variant encoded by AB384703 is identical to the variant used...
by Nakatsu et al. (22), containing an N-terminally extension of 58 amino acids compared to common gene bank entries, thereby comprising 2103 amino acids in total. These N-terminal 58 amino acids were implicated in dynamic shuttling of PI4KIIIα to the plasma membrane (22) and this function should therefore be retained in the PI4KIIIα variant used in our study. Notably, amino acid 1 in P42356.3 (13) refers to amino acid 59 in the PI4KIIIα variant used throughout this study (AB384703). Construction of pTM and pWPI plasmids encoding for full-length shRNA-resistant HA-PI4KIIIα has been described before (10). PI4KIIIα truncation mutants were cloned into pTM and pWPI constructs by PCR on pTM HA-PI4KIIIα templates (primer sequences can be obtained upon request). PCR fragments of N-terminal deletions were cloned into pTM plasmids using restriction enzymes NcoI and either KasI (Δ1-1399) or EcoRI (Δ1-1794), respectively. The sequence encoding for Δ1-1101 was transferred into pTM plasmids from the corresponding pWPI plasmid using restriction enzymes Ascl, SpeI and BstEII. C-terminal deletions were cloned using EcoRI and KasI (Δ1796-2103), Sfbl (Δ1447-2103), RsrII (Δ1104-2103) or AccI (Δ802-2103), respectively. Construction of the internal deletion Δ802-1399 or Δ1948-1991 was performed by overlap PCR using following primer combinations: S_del800_1400, A_del800-1400, S_delC800AccI and A_delN1450Kas for pTM Δ802-1399 or S_DelActive, A_delActive, S_DelActiveKas and A_EcoStopPI4 for pTM Δ1948-1991, respectively. pWPI plasmids were either created by PCR or were constructed by transferring PI4KIIIα deletion sequences from pTM to pWPI plasmids using the following restriction enzymes: BsiWI, MluI and XbaI for Δ1796-2103 or BstXI and SpeI for Δ802-2103, respectively. pWPI plasmids encoding for mutants Δ1-1152, Δ1-1202, Δ1-1252 were created by overlap PCR, while primers S_del1-1152_N and A_del1-1152_C were used as flanking primers for PCRs of all three constructs. pWPI plasmids encoding for mutants ΔDII, ΔDIII, ΔHLH, ΔNLS and ΔLZ were created by overlap PCR using ScPPT and ALKUSeq as flanking primers. pWPI constructs
encoding for mutants ∆LKU and ∆PH were created accordingly by using SPI4K4730seq and A_EMCV as flanking primers. pTM plasmids encoding for mutants ∆1-1152, ∆1-1202, ∆1-1252, ∆DII, ∆DIII, ∆HLH, ∆LZ or ∆NLS were cloned using an Ncol/BstEII fragment from the respective pWPI plasmid, which was exchanged with the Ncol/BstEII fragment yielded from pTM HA-PI4KIIIα ∆1-1101, while SpeI/BstEII sites were used for cloning of pTM HA-PI4KIIIα ∆LKU or ∆PH. Oligonucleotides were purchased from Sigma-Aldrich and all PCR-derived sequences were confirmed by sequencing (GATC, Konstanz, Germany). For in vitro transcription of HCV subgenomic RNA, the construct pFKI389-Lucubi-NS3-3’/JFH1wt_δg was used as described before (37).

Transient HCV replication

Transient HCV RNA replication assays were performed as described previously (38). In brief, five µg replicon-encoding plasmid DNA harboring hepatitis delta virus ribozymes were used for in vitro transcription. Purified RNA was transfected in 4x10⁶ Huh7-Lunet cells by electroporation. Cells were resuspended in twelve ml DMEM, two ml aliquots were seeded per well of a 6-well plate and replication was determined by measuring luciferase activity at 4 h, 24 h, 48 h and 72 h post electroporation. Values obtained 4 h after transfection were used to normalize for transfection efficiency.

Immunofluorescence analysis and PI4P quantitation

For overexpression of HCV or HA-tagged proteins, Huh7-Lunet T7 cells were transfected with LT1 transfection agent (Mirus Bio LLC, Madison, WI, USA) according to manufacturer’s instructions and were fixed 24 h post-transfection. Immunofluorescence protocol was performed as described elsewhere (7). In brief: Cells were fixed in 4 % PFA for 20 min and permeabilized...
with 50 µg/ml Digitonin for 5 min for imaging of intracellular PI4P or with 0.5 % Triton-X 100 for 15 min for co-localization analysis of HA-tagged proteins with HCV NS5A. Primary antibodies were incubated in 3 % BSA for 1 h at RT. NS5A was detected by using either NS5A-specific monoclonal mouse antibody (9E10, generous gift from Charles M. Rice) with a final concentration of 3 µg/ml or using a polyclonal NS5A rabbit antiserum (#4952, (36)) at a dilution of 1:500. PI4P was stained using monoclonal mouse IgM anti-PI4P antibody (Echelon, Z-P004) with a final concentration of 5 µg/ml. HA-tagged proteins were stained using monoclonal mouse HA-antibodies (Sigma-Aldrich) at a dilution of 1:200. Alexa 488, 561 or 647 conjugated secondary antibodies (Invitrogen, Molecular Probes) were incubated in 3 % BSA for 45 min at RT with a dilution of 1:1000. Nuclei were stained using DAPI for 1 min at a dilution of 1:4000 after incubation with secondary antibodies. Cells were mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, USA) and whole-cell z-stacks for PI4P analysis were acquired with a Leica SP2 confocal laser-scanning microscope. Quantification of intracellular PI4P signals was described before (7). For co-localization analysis, samples were imaged with an Ultraview ERS spinning disk (PerkinElmer Life Sciences) on a Nikon TE2000-E inverted confocal microscope using a Plan-Apochromat VC 100X objective (NA 1.4). Optical sections of 0.13 µM were acquired separately for each channel. z-stacks were deconvolved with a theoretical point-spread-function and chromatical shifts between green and far-red dyes were corrected according to recordings of multi-fluorescent beads under equal conditions (Tetraspeck Beads, Invitrogen) using Huygens Essential software (SVI, Netherlands). 3D co-localization of fluorescence signals was evaluated quantitatively for Pearson's correlation coefficient by using the integrated function in Huygens Essential.
Metabolic radiolabeling of proteins, immunoprecipitation and Western blotting

Metabolic labeling and immunoprecipitation of NS5A JFH-1 or HA-PI4KIIIα was described earlier (10). Polyclonal sheep antibodies directed against NS5A of JFH-1 were a generous gift of M. Harris (Leeds University, U.K.), monoclonal mouse antibodies against HA-tag were obtained from Sigma-Aldrich (H3663). For Western blotting, 1/10 of cells of a T25 cell culture flask were denatured and heated in 2x Laemmli-buffer and loaded onto an 8 % polyacrylamide-SDS gel. After separation and transfer to a PVDF membrane, PI4KIIIα was detected using monoclonal rabbit antibodies (Cell-Signaling). HA-tagged proteins or β-actin were detected using monoclonal mouse antibodies (Sigma H3663 or A5441, respectively).

In vitro lipid kinase assay after immunoprecipitation of HA-tagged PI4KIIIα deletion variants

In vitro lipid kinase assays were performed as described previously by Berger et al (33). Huh7-Lunet T7 shPI4K cells were seeded into a 10 cm dish and were transfected with HA-PI4KIIIα deletion variants. Twenty-four hours post transfection, cells were washed once with PBS and lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 10 % glycerol and protease inhibitor cocktail (Roche)) on ice for 1 hour. After clearing of the lysate (14,000 rpm, 45 min, 4 °C), proteins were immunoprecipitated overnight using agarose beads coupled to HA-specific antibodies (Sigma-Aldrich). Beads were washed twice with lysis buffer, once with PBS and once with lipid kinase assay buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.5 mM EGTA, 0.4 % Triton-X 100). Beads were resuspended in 1 ml lipid kinase assay buffer and 300 μl were used for Western blot analysis. The remaining 700 μl were spun down and resuspended in 35 μl lipid kinase assay buffer. For measurement of in vitro lipid kinase activity, the following reaction mix was prepared: 10 μM phosphatidylinositol/phosphoserine (PI/PS,
Invitrogen), 10 μM ATP (Roche), 2 mM DTT, 10 μCi [γ-32P]ATP (Hartmann Analytik) and lipid kinase assay buffer in a final volume of 50 μl. The reaction was started with the addition of 10 μl bead slurry and incubated for 1 h at room temperature in a shaker. The reaction was stopped with the addition of 200 μl 1 M HCl and lipids were extracted by the addition of 400 μl chloroform/methanol (1:1) suspension. After vortexing and centrifugation (13,000 rpm, 3 min), 125 μl of the lower organic phase were transferred to a scintillation counting vial and 4 ml of Ultima Gold (PerkinElmer) were added. Incorporation of [32P] was measured by using a liquid scintillation counter and is expressed as counts per minute (cpm).

**Statistical analysis and software**

Statistical analyses as indicated in the corresponding figures were performed using Microsoft Excel and GraphPad Prism software. Fluorescence images were analyzed using ImageJ/Fiji and Huygens Essential software. Figures were arranged with Adobe Photoshop and Adobe Illustrator.

**Homology modeling of PI4KIIIα Δ1-1535**

The delimitation of the PI3K-like region of PI4KIIIα was obtained by a sequence alignment with PSI-BLAST (39) against the sequence of the human PI4KIIIα. Only PI3K with known structures were taken into account. In turn, a multiple alignment was generated with those results using COBALT (40). This led to a reliable alignment with structural templates matching PI4KIIIα residues in the region 1536-2102. I-TASSER (41) was used to generate a structural model of PI4KIIIα Δ1-1535. The most relevant model was obtained with the Drosophila PI3KIII VPS34 as template (PDB code 2X6F) (42).
Sequence-based domain predictions of PI4KIIIα Δ1536-2102

We used the QUICK2D web service (http://toolkit.tuebingen.mpg.de/quick2_d) from the MPI Bioinformatics Toolkit (43) to perform secondary structure and disorder analysis on the N-terminus of the PI4KIIIα sequence. QUICK2D runs parallel analyses with the most accurate secondary structure and disorder prediction programs. The reliability of the predictions was evaluated based on the agreement between different programs.

Results

PI4KIIIα interacts with NS5A via multiple interaction sites.

In this study, we aimed to investigate structural requirements of PI4KIIIα enzymatic function and the requirements for HCV replication. Previously, we reported a crucial role of NS5A-PI4KIIIα interaction in HCV replication by identifying NS5A residues important for PI4KIIIα interaction and recruitment (10). Here, we first aimed to identify the binding site between NS5A and PI4KIIIα by constructing different N- and C-terminal truncations of PI4KIIIα that were tested for NS5A interaction by co-immunoprecipitation. We employed three serial deletions of the N-terminus (Δ1-1101, Δ1-1399, Δ1-1794), four serial deletions of the C-terminus (Δ1796-2103, Δ1447-2103, Δ1104-2103, Δ802-2103) and two internal deletions (Δ802-1399, Δ1948-1991), encompassing functional motifs assigned in a previous study (Fig. 1A, (13)). All mutants were expressed under transcriptional control of a T7 promoter and were co-transfected with a plasmid encoding NS5A of isolate JFH-1 into Huh7-Lunet cells stably expressing T7 RNA polymerase (Huh7-Lunet T7) followed by immunoprecipitation of NS5A or HA-tagged proteins. All PI4KIIIα mutants were expressed in significant amounts, but very little NS5A co-precipitated in
the HA-IP (Fig. 1B, right panel). This was in line with previous results and likely due to the much higher NS5A expression levels (10). However, when using NS5A-specific antibodies, all PI4KIIIα mutants co-precipitated with NS5A (Fig. 1B, left panel, and C) and the specificity could not be increased by changing IP conditions (data not shown). Co-precipitation efficiency was reduced for an NS5A variant harboring a mutation in the PI4KIIIα interaction site (mutHIT (10)), but not to the same extent as the same mutant expressed in the context of NS3 to NS5B ((10), data not shown). Indeed, when NS5A was expressed in the context of the NS3-5B polyprotein, co-precipitation seemed to be more specific and the efficiency was significantly reduced for two deletion mutants (Δ1-1794 and Δ1104-2103, respectively; Fig. 1D). Based on these data still several regions within PI4KIIIα seemed to independently contribute to the interaction with NS5A. However, we regarded it unlikely that all of them would contribute to the function of PI4KIIIα in HCV replication, since we had been able to assign a single 7-residue major functional PI4KIIIα interaction motif in NS5A (10). Therefore, we sought for alternative ways to characterize regions of PI4KIIIα functionally important for HCV replication.

Deletion of the N-terminal 1101 amino acids of PI4KIIIα has no effect on HCV replication.

To identify regions within PI4KIIIα important for HCV, we analyzed a set of PI4KIIIα mutants for their ability to support HCV replication (Fig. 1A, marked with asterisks) including a full-length PI4KIIIα variant carrying a point mutation at position 1957, thereby rendering the protein enzymatically inactive (D1957A (7)). HA-tagged shRNA-resistant PI4KIIIα variants were expressed in Huh7-Lunet cells with a constitutive knockdown of endogenous PI4KIIIα (shPI4K) using selectable lentiviral vectors (Fig. 2B). Endogenous PI4KIIIα was found at very low amounts in the control cell line expressing a non-targeting shRNA (NT, Fig. 2B, black arrowhead) and was absent in the shPI4K cell line. All PI4KIIIα mutants were expressed at
higher levels than endogenous PI4KIIIα in the NT cell line. Subgenomic JFH-1 firefly luciferase reporter replications were used to analyze the ability of PI4KIIIα mutants to support HCV replication (Fig. 2A). Knockdown of endogenous PI4KIIIα resulted in about 100-fold reduction of HCV replication as compared to the NT cell line at all measured time points, which was rescued by expression of PI4KIIIα wt but not of the inactive D1957A mutant, as expected (Fig. 2C). Interestingly, HCV replication efficiency was not enhanced beyond the level of the control cells, despite strongly enhanced PI4KIIIα expression levels in cells ectopically expressing the full-length enzyme (Fig. 2B, lower panel, NT compared to wt), suggesting that low amounts of active PI4KIIIα were already sufficient to achieve maximal HCV replication efficiency. Remarkably, a cell line expressing a PI4KIIIα mutant lacking the N-terminal 1101 amino acids (Δ1-1101) was still able to support HCV replication to the same extent as the NT or PI4KIIIα wt cell line. All other PI4KIIIα variants were not able to rescue HCV replication. Still, a limited increase in HCV replication compared to the shPI4K or D1957A cell line was observed for some of the mutants, including C-terminal deletions lacking the catalytic center of the lipid kinase. This might argue for an indirect role of other structural features of PI4KIIIα in HCV replication or for subtle differences in PI4KIIIα knockdown efficiency in those cell lines. Altogether, these findings demonstrated that deletion of the N-terminal 1101 amino acids did not compromise the functions of PI4KIIIα in HCV replication, whereas further truncations of the N-terminus or all deletions in the C-terminal half of PI4KIIIα were deleterious.

Deletion of the N-terminal 1101 amino acids has no effect on PI4KIIIα enzymatic activity.

Our previous studies had shown that HCV replication or expression of HCV NS3 to NS5B activated lipid kinase activity of PI4KIIIα, resulting in substantially increased amounts of intracellular PI4P (7,10). Therefore, increased intracellular PI4P levels in presence of HCV can
be used as an indirect measure of intracellular PI4KIIIα activity and for a functional interaction with HCV proteins. Thus, we characterized the same set of shPI4K Lunet-T7 cell lines stably expressing the PI4KIIIα mutants for the induction of PI4P in presence of HCV. Since most of the mutants did not support HCV replication (Fig. 2C), we ectopically expressed the NS3 to NS5B polyprotein under transcriptional control of the T7 promoter. In mock-transfected NT cells, PI4P localized mainly to the Golgi compartment in a perinuclear area, as reported previously (Fig. 3A (7,10,23)). Knockdown of PI4KIIIα did not have any effects on the distribution or the amount of PI4P as expected, since these PI4P pools are mainly synthesized by the related PI4KIIIβ isoform (16), Fig. 3A, mock shPI4K). In NT cells expressing HCV NS3 to NS5B, PI4P was found in high amounts at intracellular membranes all over the cell, only partially co-localizing with HCV NS5A (Fig. 3A) and total PI4P amounts were four- to five-fold increased compared to mock-transfected cells (Fig. 3B). Knockdown of PI4KIIIα abolished the HCV-dependent induction of these PI4P pools (Fig. 3B) and also altered the subcellular distribution of NS5A to a more “clustered” morphology (Fig. 3A, pTM NS3-5B JFH-1, shPI4K, (7)). Both phenotypes – induction of PI4P as well as localization of NS5A – were rescued in cell lines expressing PI4KIIIα wt (Fig. 3A, B).

Among the PI4KIIIα deletion mutants, only cell lines harboring Δ1-1101 exhibited high intracellular PI4P pools and NS5A distribution comparable to the NT cell line and to PI4KIIIα wt expression, suggesting that the N-terminal 1101 amino acids of PI4KIIIα were dispensable for the activation of PI4KIIIα by the HCV nonstructural proteins. To address the impact of the deletions on enzymatic activity of PI4KIIIα more directly, we analyzed whether these mutants exhibited lipid kinase activity in vitro. HA-tagged PI4KIIIα protein variants were transiently expressed under transcriptional control of the T7 promoter in shPI4K cells constitutively expressing T7 RNA polymerase to exclude any cross-contamination with endogenous PI4KIIIα and were purified by immunoprecipitation using HA-specific antibodies (Fig. 3C, upper panel) and
subjected to an *in vitro* lipid kinase assay. Expression levels (Fig. 3C, lower panel) and purification efficiency varied to some extent among different constructs, still, in this experiment, all PI4KIIIα variants except D1957A and Δ1796-2103 could be recovered in sufficient amounts (Fig. 3C, upper panel). Again, only PI4KIIIα wt and the Δ1-1101 mutant showed *in vitro* lipid kinase activity above background levels (Fig. 3D).

Collectively, our results indicated that the N-terminal 1101 amino acids of PI4KIIIα were not required for enzymatic activity of PI4KIIIα or for functional interaction with HCV, whereas larger N-terminal deletions (Δ1-1399) or deletions within the C-terminus were not tolerated.

Subcellular localization of HA-PI4KIIIα mutants and recruitment by NS5A.

PI4KIIIα has been described to primarily contribute to PI4P synthesis in the ER and the plasma membrane, in contrast to other PI4K isoforms (reviewed in (16)). Therefore, we next analyzed the subcellular localization of the PI4KIIIα deletion mutants to identify determinants of subcellular localization by transient transfection of T7-driven constructs (Fig. 4A). Interestingly, PI4KIIIα wt as well as all mutants were distributed broadly throughout the entire cytoplasm of the transfected cells, and did not significantly co-localize with the ER-marker PDI or a Golgi marker (Fig. 4A and data not shown), in line with a recent report (22). Only the PI4KIIIα variant with the largest N-terminal deletion (Δ1-1794) had a more prominent nuclear localization (data not shown), which might be either due to a loss of a nuclear export signal or, more likely, because of the small size of this variant (ca. 30 kDa), allowing diffusion through the nuclear pore (44).

In essence, although we have generated a set of huge, non-overlapping deletions of PI4KIIIα, all mutants showed almost the same broad subcellular distribution and no clear co-localization to particular intracellular membranes.
We have shown before that PI4KIIIα is recruited to the MW, which might be crucial for HCV replication (7). Therefore, we co-expressed NS3 to NS5B with the different HA-tagged PI4KIIIα mutants and found that PI4KIIIα wt and the inactive D1957A point mutant widely co-localized with NS5A in distinct foci throughout the cell, arguing for a specific recruitment of PI4KIIIα independent of enzymatic activity. Importantly, only mutant Δ1-1101 co-localized to NS5A-positive compartments in the same way as PI4KIIIα wt, whereas all other deletion mutants showed no specific recruitment to NS5A at all (Fig. 4B, C). These data indicate that recruitment of PI4KIIIα to NS5A-positive compartments seemed not to depend on a distinct interaction region but rather relied on an overall structurally intact kinase.

In summary, gross mapping of functionally important regions of PI4KIIIα revealed that the N-terminal 1101 amino acids were dispensable for enzymatic activity, subcellular localization, specific recruitment to the HCV replication sites and HCV replication. In contrast, mutants with further N-terminal deletions and all C-terminal deletions had lost enzymatic activity, recruitment by HCV and failed to support HCV replication (Table 1).

**N-terminal deletions up to 1152 amino acids do not compromise major PI4KIIIα functions.**

Our previous experiments clearly revealed that the region between residues 1102 and 1399 was critical for the enzymatic function of PI4KIIIα as well as for its role in HCV replication. Therefore, we next aimed to define more precisely the N-terminal boundary critical for PI4KIIIα function. We constructed deletion mutants lacking the N-terminal 1152, 1202 or 1252 amino acids, respectively, and stably (Fig. 5A-C) or transiently (Fig. 5D-F) expressed these mutants in shPI4K cells. We analyzed these cell lines for their ability to support HCV replication (Fig. 5B), for the activation of enzymatic activity by HCV NS3 to NS5B (Fig. 5C) and for enzymatic activity *in vitro* (Fig. 5D, E). All functions were fully retained by mutant Δ1-1152, whereas
mutants Δ1-1202 and Δ1-1252 showed dramatically reduced or no rescue of HCV replication, respectively (Fig. 5B), no significant induction of intracellular PI4P levels upon expression of NS3 to NS5B (Fig. 5C) and no significant lipid kinase activity in vitro (Fig. 5E), as also observed for other enzymatically inactive mutants (compare to Fig. 2 and 3, Table 1). Although mutant Δ1-1202 was devoid of any detectable enzymatic activity in vitro it still rescued HCV replication and intracellular PI4P induction to some extent, arguing for a minimal residual activity that might be sufficient for limited HCV replication. Subcellular localization of all three mutants was not distinguishable from PI4KIIIα wt in absence of HCV (data not shown), but recruitment to NS5A-positive compartments was completely lost for mutants Δ1-1202 and Δ1-1252, whereas co-localization of mutant Δ1-1152 with NS5A-positive dots was slightly but significantly reduced compared to mutant Δ1-1101 (Fig. 5F), suggesting that region 1102-1202 might play a role in specific recruitment of PI4KIIIα to the HCV replication sites.

Conclusively, we found that the N-terminal 1152 amino acids of PI4KIIIα are not required for lipid kinase activity or HCV replication.

Functional analysis of subdomains and predicted motifs in the C-terminal region of PI4KIIIα.

After definition of the N-terminal boundaries, we next focused on C-terminal motifs of PI4KIIIα and their contribution to HCV replication and enzymatic activity. Based on the domain annotations by Gehrmann et al. (13) we individually deleted the nuclear localization signal (ΔNLS), the helix-loop-helix motif (ΔHLH), the leucine zipper (ΔLZ), the lipid kinase unique domain (ΔLKU) or the PH domain (ΔPH, Fig. 1A, 6A). We additionally included deletions encompassing residues 1280-1430 (designated DII) or 1430-1530 (designated DIII, Fig. 9A), respectively, since these regions were predicted to fold into distinct alpha-helical subdomains (Fig. 9A and B). We chose the N-terminally truncated mutant Δ1-1101 with an N-terminal HA-
tag as backbone for all further deletions of C-terminal domains (Fig. 6A) since this mutant was identical to the full-length enzyme regarding recruitment by HCV, rescue of HCV replication and induction of PI4P synthesis by HCV and similar regarding enzymatic activity. Using this truncated variant facilitated expression and detection of the mutants, although we cannot fully exclude that some deletion mutants might behave differently in the context of the full-length enzyme.

To address their ability to rescue HCV replication, mutants were expressed in shPI4K cells by lentiviral transfer (Fig. 6B) and HCV replication was assessed as before by transfection of subgenomic JFH-1 luciferase reporter replicons using the cell line expressing Δ1-1101 as a positive control (Fig. 6C). Only the mutant lacking the proposed NLS was still able to fully support HCV replication comparable to the Δ1-1101 mutant or the NT cell line, whereas expression of all other mutants did not substantially rescue HCV replication, apart from some minor increases similar to those observed before (Fig. 6C, compare to Fig. 2C). Thus, the region encompassing the NLS seemed to be dispensable for HCV replication. Similar results were obtained by analysis of the ability of the mutants to give rise to increased PI4P levels upon expression of HCV NS3 to 5B (Fig. 7A, B) and lipid kinase activity in vitro (Fig. 7C, D). Again, all mutants except ΔNLS were inactive. However, stimulation of PI4P synthesis in cell culture (Fig. 7A, B) as well as lipid kinase activity (Fig. 7C, D) of ΔNLS was strongly reduced compared to the parental Δ1-1101 mutant. These findings demonstrate that deletion of the NLS partly compromised enzymatic activity of PI4KIIIα, which, however, was still sufficient to fully support HCV replication.

We next examined whether the deletions might affect intracellular distribution of PI4KIIIα. As observed for the larger deletions described above, all mutants again localized broadly throughout the cytoplasm in absence of HCV proteins, similar to the parental Δ1-1101 mutant (Fig. 8A and
data not shown). Hence, we were not able to identify regions that are solely responsible for correct localization of PI4KIIIα. In contrast, all deletions affected co-localization with NS5A upon co-expression of NS3 to NS5B, albeit to various extent (Fig. 8B, C). Deletion of residues 1280-1430 (DII) or 1430-1530 (DIII) in the context of Δ1-1101 resulted in a complete loss of NS5A co-localization (Fig. 8B and C), arguing for an important role of these regions in recruitment of PI4KIIIα by HCV or for the structural integrity of the enzyme. Deletions of smaller motifs within DII and DIII (ΔNLS, ΔHLH, ΔLZ) affected NS5A co-localization to various extents, probably rather reflecting the impairment of the overall structure than targeting a specific interaction motif with HCV proteins. However, mutants ΔLKU and ΔPH still partly co-localized with NS5A, despite their lack of enzymatic activity, suggesting that these regions are not essential for recruitment by the HCV replicase.

Taken together, our functional studies on the C-terminal half of PI4KIIIα demonstrated a tight linkage between rescue of HCV replication, PI4P induction and lipid kinase activity in vitro (Table 1). All deletions analyzed in the context of Δ1-1101 except ΔNLS entirely abrogated enzymatic activity, suggesting that the entire C-terminal half of PI4KIIIα (aa 1152-2103) is involved in generating the catalytically active enzyme and that any gross deletion in this region might be deleterious for structural integrity. Recruitment of PI4KIIIα by the HCV proteins was not entirely abrogated by some of the deletions, suggesting that this process was mediated by different structural elements at the C-terminal half.

Homology modeling of the C-terminus of PI4KIIIα allows refinement of domain annotations.

Structural information of domains at the C-terminal part of PI4KIIIα was not available, but these domains seemed to play important roles in enzymatic function and recruitment by HCV.
Therefore, we used secondary structure predictions and structural modeling of residues 1102-2102 to identify motifs and domains, which might be involved in those functions (summarized in Fig. 9A). The region encompassing residues 1101-1535 has no detectable homology to any protein of known structure, even using the most sensitive detection and structure prediction algorithms available (45). However, based on secondary structure prediction, it is likely almost all helical. Furthermore, there are clear breaks in the predicted secondary structure associated with a strong propensity to disorder in the sequences flanking residues 1130-1270 (Fig. 9B). This suggests that there is a distinct α-helical domain or subdomain in this region, which we termed DI. Breaks are less obvious in the 1271-1535 stretch, but there may be two more largely α-helical (sub)domains DII and DIII further downstream (not shown).

For modeling the very C-terminus of PI4KIIIα, we used structural information of phosphoinositide 3-kinases (PI3K), which are distant homologs of type III phosphoinositide 4-kinases (46), a relationship that is reflected by their common sensitivity to wortmannin. Shortly after Gehrmann et al. identified and cloned human PI4KIIIα (47), the first crystal structures of PI3Ks became available (48). Using PI3K as templates it is thus possible to accurately model the C-terminal part of PI4KIIIα for residues 1536-2102, with the exception of residues 1810-1840 that constitute an insertion in PI4KIIIα compared to PI3K (Fig. 9C, marked with an asterisk). As for PI3K (48), the PI4KIIIα 1536-2102 model is best described as an armadillo repeat (ARM) domain (aa 1536-1723, encompassing LKU) followed by a two-lobed kinase domain (N-lobe: aa 1724-1900; C-lobe: aa 1901-2102, Fig. 9C). The N-lobe’s N-terminus thus corresponds to the region ascribed by Gehrmann et al. as a PH domain (13), although it lacks the two N-terminal strands and the C-terminal capping helix of the pleckstrin fold. Instead, it is flanked by two helices at its N-terminal end and one helix and two strands at its C-terminal end. The last strand terminates in a loop that crosses to the C-lobe and provides the floor to the ATP- and
wortmannin-binding pocket that is flanked by both lobes (Fig. 9D). The C-lobe harbors the catalytic residues including D1957 (Fig. 9D) and the “activation loop” (Fig. 9D, represented in magenta) that is involved in PI recognition and contributes to determining phosphoinositide specificity (48). Relative to the catalytic residues and the activation loop (and thus the ER membrane), the 1810-1840 insertion lies on the opposite face of PI4KIIIα next to a region that in PI3K is occupied by regulatory subdomains (Fig. 9C, region represented as yellow ellipse).

Conclusively, in this study we provide an updated view on the C-terminus of PI4KIIIα (summarized in Fig. 9A) that could guide further studies on the roles of different C-terminal domains in the function of PI4KIIIα and also of related lipid kinases.

Discussion

In this study, we investigated structural requirements of PI4KIIIα for HCV replication and enzymatic activity. Therefore, we generated a panel of PI4KIIIα deletion mutants and characterized them regarding their enzymatic activity, their ability to be activated by expression of the HCV polyprotein, their efficiency in rescuing HCV replication, their subcellular localization in presence and absence of HCV proteins and their interaction with HCV NS5A. We found a very close linkage between enzymatic activity, induction of PI4P and rescue of HCV replication, in line with previous results of us and others, suggesting that the catalytically active center of PI4KIIIα is essential for its role in HCV replication (10,15). These data were largely connected to the ability of PI4KIIIα to be recruited to the HCV replication sites. In contrast, most mutants containing gross non-overlapping deletions still physically interacted with NS5A and showed a subcellular distribution unaltered from the full-length protein in absence of HCV, arguing for several interaction sites and redundant determinants for PI4KIIIα localization, not all of them being essential for function.
Our previous studies have shown that PI4KIIIα physically interacted with HCV NS5A and NS5B (7,10) and identified a region at the C-terminus of NS5A domain I, which is critically involved in the functional interaction of NS5A and PI4KIIIα and is required for activation of PI4KIIIα (10). We now aimed to identify the specific interaction region within PI4KIIIα, but our results indicate that several redundant sites of physical interaction between PI4KIIIα and NS5A exist, which not all might be functionally important. A previous study has shown that PI4KIIIα interacted with NS5A through amino acids 400-600 (35), but it seems very unlikely that this interaction is required to support HCV replication, since deletion of this region did not compromise the ability to rescue HCV replication (Δ1-1101, Table 1). Since literally all deletion constructs we have analyzed still co-precipitated to some extent with NS5A (and NS5B, data not shown), it is hard to assign a clearly defined region involved in functional HCV interaction. Interestingly, we found some discrepancy between sole expression of NS5A compared to NS3-5B. This might point to a modulation of the NS5A-PI4KIIIα interaction by NS5A phosphorylation, since proper phosphorylation of NS5A requires expression of at least NS3-5A as a polyprotein (49,50). Modulation of NS5A interaction by phosphorylation has been demonstrated for VAP-A (51) and might be a reasonable concept for PI4KIIIα, since in a previous study we have shown that PI4KIIIα directly or indirectly modulates NS5A phosphorylation (10). Although we cannot get to a final conclusion, some evidence points to an important role of the region 1102-1152 in mediating the functional interactions between NS5A and PI4KIIIα since mutant Δ1-1152 was already slightly impaired in specific recruitment to the HCV replication sites, while still retaining full enzymatic activity and the ability to rescue HCV replication. Interestingly, specific recruitment was lost for many mutants lacking enzymatic activity (Table 1), suggesting that a functional interaction with HCV proteins might not only require specific sequence motifs, but rather a properly folded PI4KIIIα enzyme. Still, enzymatic activity was not required for
recruitment/interaction, since the inactive PI4KIIIα point mutant D1957A interacts and co-localizes with NS5A as the wt enzyme. These findings suggest that recruitment of PI4KIIIα by NS5A to defined compartments precedes stimulation of kinase activity. Clearly, a more subtle mutational analysis of the C-terminal half of PI4KIIIα will be required to unravel the sites important for functional interaction with NS5A.

PI4KIIIα has been identified as an essential host factor of HCV RNA replication by a multitude of studies (7,11,27-32), but still the precise mechanism of action remains to be determined. It remains difficult to dissect specific functions of PI4KIIIα in HCV replication, since all features of PI4KIIIα we assessed in this study were tightly linked. All mutants analyzed in this study containing detectable lipid kinase activity gave rise to increased intracellular PI4P levels and were able to rescue HCV replication. These data suggest that HCV exploits the general regulatory mechanisms governing the lipid kinase activity of PI4KIIIα, which have not been identified yet (16). In addition, our data clearly show that PI4KIIIα – albeit essential for viral replication – is not a limiting factor in Huh-7 cells, since already low endogenous amounts of PI4KIIIα were sufficient to allow maximal viral replication efficiency, which could not be further increased by ectopic overexpression.

Besides analyzing aspects regarding HCV replication, for the first time our study provides a comprehensive deletion analysis to define functional domains of PI4KIIIα important for enzymatic activity. Earlier studies identified an enzymatically active mutant, which lacked the N-terminal 872 amino acids, while deletion of 1189 amino acids entirely abrogated enzymatic function (corresponding to residues 931 and 1249, respectively, in our PI4KIIIα gene), arguing for a 130 kDa enzyme as a minimal requirement for enzymatic activity (20,52,53), whereas an alternative splice variant of PI4KIIIα lacking 1249 N-terminal amino acids was shown to be inactive and did not support HCV replication (15). Our deletion analysis now revealed that in fact
the N-terminal 1152 amino acids are dispensable for enzymatic activity, whereas further deletion of 50 amino acids resulted in an inactive protein (Δ1-1202). These results more closely define the PI4KIIIα enzymatic core to a size of ca. 105-108 kDa, which is surprisingly similar to related enzymes like PI3Ks (48). Interestingly, we did not find evidence for a function of the N-terminal half of PI4KIIIα in any of our assay systems regarding subcellular localization, enzymatic activity and stimulation by HCV. A previous study has identified the very N-terminal 58 amino acids being involved in dynamic shuttling of PI4KIIIα to the plasma membrane (22), a sequence that is missed in most Genbank entries. However, since we used a construct of identical length as Nakatsu et al., plasma membrane shuttling should be retained with our full-length enzyme, but lost for Δ1-1101, thereby indicating that this mechanism is not essential for enzymatic activity or HCV replication. Surprisingly, all of our deletion mutants showed a subcellular distribution comparable to the full-length enzyme in absence of HCV proteins. The majority of signal was broadly localized throughout the cytoplasm in line with published data (18,22). Even the very N-terminus (Δ802-2103) as well as the very C-terminus (Δ1-1794) showed no gross change in subcellular localization compared to the full-length protein. We therefore were not able to assign a specific sequence within PI4KIIIα responsible for subcellular localization. In previous reports, we and others found a more distinct localization of the endogenous kinase in punctuate structures throughout the cytoplasm, consistent with an ER-association (7,16,21). In brain cells PI4KIIIα was prominently detected in the nucleus and nucleolus (21). The subcellular localization might therefore depend on cell type and expression level of PI4KIIIα. Although the majority of PI4KIIIα has been found associated with membranes (18), the determinants of membrane association have not been found yet for the mammalian enzymes (16). In the light of the very recent observation of a dynamic recruitment of PI4KIIIα to the plasma membrane, mediated by a protein complex containing EFR3 (22), it is tempting to speculate that PI4KIIIα is recruited to the
ER as well by auxiliary factors, e.g. VAP-B (22), and not by an intrinsic function of the kinase. In this scenario, overexpression as in case of our experiments might overload the membrane recruitment complex, resulting in a broad pleiotropic localization. In line with this idea, the majority of PI4KIIIα was recruited to NS5A and vanished from the cytoplasm upon co-overexpression of HCV NS3 to NS5B, which forms an abundant membrane-associated complex interacting with the kinase, thereby probably competing with the cellular recruitment complex. However, further experimentation is required to prove this hypothesis including the assessment of the membrane-associated fractions of PI4KIIIα wt and deletion mutants, as well as a proteomic analysis of potential PI4KIIIα interaction partners in the ER.

Since our N-terminal deletion mutant Δ1-1101 was comparable to the full-length enzyme in all of our assay systems, we focused our further deletion mapping on domains and motifs predicted to be located in the C-terminal half of PI4KIIIα (14). Although our experimental models were set out to differentiate between different functions, we again found a tight linkage of almost all phenotypes except subcellular localization in presence of HCV proteins. With the exception of ΔNLS, which was only moderately impaired in enzymatic activity, all other deletion mutants were completely devoid of lipid kinase activity in vitro and activation by HCV. While this result might be expected for large deletions like LKU, it seems very unlikely that short motifs like HLH or LZ are essential for the enzymatic function. HLH and LZ in particular are supposed to have a role in recognition of DNA sequences, probably for the subfraction of PI4KIIIα located in the nucleus (54). The loss of function associated with all N-terminal mutants beyond aa 1152 and internal deletions as well as the impaired function of the ΔNLS mutant might rather point to the fact that these predicted motifs are located in regions relevant for the overall structure of an active enzyme. Therefore, our deletion analysis does not rule out the existence of these predicted motifs and more subtle point mutations will be required to distinctively address their role for enzymatic
function. Still, the results of our deletion analysis is in favor of our structural model, which is based on homology to the known structure of PI3K (48) and predictions (Fig. 9). This model suggests that the region previously designated PH-domain builds the N-terminal lobe of the catalytic core, whereas the LKU domain forms an armadillo repeat. Armadillo domains mediate protein-protein interactions and are scaffold domains. Thus, in PI3K they buttress the membrane-interacting C2 domain and non-catalytic subunit. Our data furthermore suggest that the region between aa 1152-1530 is in wide parts essential for enzymatic activity and might be organized in three distinct alpha helical subdomains (Fig. 9). Disruption of these secondary structures by deletion as well as N-terminal truncations beyond aa 1152 and even deletions of smaller parts in DIII resulted in a complete loss of enzymatic activity. Only the residual activity found with ΔNLS argues for some structural flexibility in DII. It is tempting to speculate that domains DI to DIII are involved in the regulation of enzymatic activity. Allosteric regulation of kinase activity has been described before for the PI3K regulatory subunit p85 that binds and stabilizes its catalytic subunit p110 (55). Upon stimulation, e.g. by phosphorylation or by binding of phosphoproteins to SH2 domains of p85, enzymatic activity of the p110 subunit is increased (56). Binding of interaction partners like ADP-ribosylation factor also stimulates functions of the related PI4KIIIβ isoform (57). Activation by phosphorylation also has been demonstrated for PI4KIIIβ that is phosphorylated by protein kinase D at a highly conserved motif resulting in stimulation of lipid kinase activity (58). Interestingly, the phosphorylated residue lies in an insertion between the PI4KIIIβ ARM and N-CAT that in the PI4KIIIA model is right next to the 1810-1840 insertion (Fig. 9C). In PI3Ks, this region opposite the membrane and below the floor of the catalytic site (Fig. 9C, yellow ellipse) is occupied by regulatory domains. For instance, the Ras-binding domain (RBD) of PI3Kγ is located in this region. Binding of Ras across RBD and C-CAT induces a conformational change involving both membrane-interacting and catalytic regions.
of PI3Kγ, suggesting an allosteric mechanism in the kinase activation (59). PI4KIIIα may be similarly recruited to membranes and activated, although this assumption requires further experimental validation. With respect to specific recruitment of PI4KIIIα by HCV rather than recruitment of PI4KIIIβ, it is interesting to note that PI4KIIIβ does not have a region N-terminal to its ARM domain similar to DI-DIII of PI4KIIIα.

Taken together, with this study we characterized PI4KIIIα in depth as an essential HCV host factor and defined requirements for HCV replication and enzymatic activity as well as providing insights into the structural complexity that mediates kinase function. We provide a structural model of the catalytic center of the enzyme and thereby lay the ground for more detailed reverse genetic analyzes to define more precisely the functions of individual PI4KIIIα subdomains.

Acknowledgements

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Reference List


Table 1: Summary of phenotypes observed for the different PI4KIIIα mutant variants.

<table>
<thead>
<tr>
<th>PI4KIIIα mutation</th>
<th>NS5A interaction*</th>
<th>NS5A interaction (NS3-5B)*</th>
<th>HCV replicationb</th>
<th>PI4P inductionc &amp; in vitro lipid kinase activityd</th>
<th>Recruitment to membranous web e</th>
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Plus-signs are indicated for the following criteria:

* NS5A interaction is not significantly impaired compared to wt PI4KIIIα (Fig. 1 and S1).

b: Rescue of HCV replication is not significantly impaired compared to the NT cell line (Fig. 2, 5 and 6).

c: Intracellular PI4P is significantly increased compared to mock-transfected cells (Fig. 3 and 7).
In vitro lipid kinase activity is at least 10% of the corresponding positive control (Fig. 3 and 7).

Pearson correlation is significant over the number of analyzed samples (Fig. 4, 5 and 8). +/− denotes mutants that do not show significant Pearson correlation values, but showed substantially higher correlation than other mutants in the respective experiments.

*: adapted from (10)
n.a.: not analyzed

n.a.: not analyzed
Figure 1: PI4KIIIα interacts with NS5A via multiple interaction sites. (A) Schematic representation of HA-tagged, truncated PI4KIIIα proteins used for NS5A interaction mapping and subsequent analyzes. Annotations of subdomains and motifs were adapted from Gehrmann et al. (13) and are indicated with the respective amino acids positions. Residues encompassing the respective truncations are indicated at the right. Asterisks denote mutants used for further functional characterization. All numberings refer to GenBank accession number AB384703. (B, C, D) Plasmids encoding for different HA-PI4KIIIα truncations were co-transfected with constructs encoding for HCV NS5A (B, C) or NS3-5B (D) into Huh7-Lunet T7 cells. Proteins were metabolically labeled with [35S]-Methionine/Cysteine and immunoprecipitated using polyclonal antibodies directed against NS5A or monoclonal antibodies directed against the HA-peptide, respectively. Proteins were separated by 10% SDS-PAGE and visualized by autoradiography. (C, D) Bands obtained from experiments as shown in B (C) were quantified by densitometry. Pulldown efficiency of PI4KIIIα mutants by NS5A is shown as ratio of total amounts of HA-PI4KIIIα to co-precipitated protein by NS5A. Shown are mean values and standard deviations of three different experiments.

Figure 2: Deletion of the N-terminal 1101 amino acids of PI4KIIIα does not compromise its function in HCV replication. (A) Schematic representation of the subgenomic firefly luciferase reporter replicon of HCV genotype 2a (isolate JFH-1) used in this study. (B) Huh7-Lunet T7 were stably transduced with selectable lentiviral vectors expressing non-targeting shRNA (NT) or shRNA targeting endogenous PI4KIIIα (shPI4K) and encoding HA-tagged PI4KIIIα variants as indicated. Knockdown of endogenous PI4KIIIα and ectopic expression of HA-tagged PI4KIIIα
variants was assessed by Western Blot using monoclonal antibodies against the HA-peptide (top panel) or polyclonal antibodies against PI4KIIIα (middle panel), respectively. Asterisks indicate PI4KIIIα variants which have lost epitopes recognized by the polyclonal antiserum. An arrowhead points to endogenous PI4KIIIα. (C) Replication of HCV was quantified by transfection of firefly luciferase reporter replicons into the indicated cell lines. Replication efficiency is expressed as relative light units (RLU) normalized to 4 h after transfection to account for transfection efficiency. A replication-deficient deletion mutant in NS5B (ΔGDD) served as negative control. Significances over shPI4K cells are shown for replication efficiencies 48 h after transfection. Data represent mean values and standard deviations of a representative experiment (n=3). ***: p < 0.001.

Figure 3: Lipid kinase activity of truncated PI4KIIIα proteins *in vitro* and activation by HCV in cell culture. (A) Plasmids encoding for HCV NS3-5B were transfected into Huh7-Lunet T7 cell lines depleted of endogenous PI4KIIIα and stably expressing the indicated variants of PI4KIIIα (for details refer to the legend of Fig. 2). 24 h after transfection, cells were fixed, permeabilized with Digitonin and NS5A (red) or PI4P (green) were stained using monoclonal antibodies, respectively. Nuclei (blue) were stained using DAPI. (B) Fluorescence signals of PI4P from whole-cell z-stacks were quantified using ImageJ software. PI4P induction was quantified by normalizing intensities of HCV-transfected cells relative to mock-transfected cells of each cell line. Standard deviations are indicated. At least 30 cells were quantified for each condition. (C) pTM constructs encoding for the indicated HA-tagged PI4KIIIα mutants were transfected into Huh7-Lunet T7 shPI4K cells. 24 h after transfection cells were lysed and HA-tagged proteins were immunoprecipitated using HA-beads. PI4KIIIα and β-actin were visualized by Western blot, using antibodies against the HA-peptide and β-actin, respectively, in the cell lysate (Input)
and after IP. An asterisk indicates the position of a weak band of the Δ1-1794 mutant. The ca. 46 kDa band in the IP fractions corresponds to the heavy chain of the antibodies used for IP. (D) Immunoprecipitated HA-PI4KIIIα mutants were subjected to in vitro lipid kinase assays based on incorporation of [32P] into phosphatidylinositol substrates. Radioactive lipids were extracted and subjected to liquid scintillation counting. Depicted are counts per minute (cpm) obtained for the different PI4KIIIα mutants after background subtraction. n.d.: not determined due to low recovery levels. Lack of enzymatic activity of mutants D1957A and Δ1796-2103 was determined in independent experiments (Fig. 5E and data not shown, respectively).

Figure 4: Subcellular localization of PI4KIIIα truncation mutants in presence and absence of HCV NS proteins. Huh7-Lunet shPI4K cells were transfected with plasmids encoding for the indicated HA-tagged PI4KIIIα variants (A, B, C) and in addition with constructs expressing HCV NS3 to NS5B (B, C). (A) Proteins were stained using monoclonal antibodies directed against PDI as marker of the ER (red) or HA-peptide (green), respectively. Nuclei (blue) were stained using DAPI. (B) HCV NS5A or HA-tagged proteins were stained using monoclonal antibodies directed against NS5A (red) or HA-peptide (green), respectively. (C) 3D co-localization of NS5A and PI4KIIIα in experiments as shown in (B) was assessed by recording whole-cell z-stacks and analyzed using Huygens Essential software. Depicted are mean values and standard deviations of Pearson correlation co-efficients of at least ten individual cells per condition. *: p < 0.05.

Figure 5: Mapping of the N-terminal border required for PI4KIIIα function. (A) Huh7-Lunet T7 were stably transduced with selectable lentiviral vectors expressing non-targeting shRNA (NT) or shRNA targeting endogenous PI4KIIIα (shPI4K) and encoding HA-tagged PI4KIIIα variants as indicated. Knockdown of endogenous PI4KIIIα and ectopic expression of
HA-tagged PI4KIIIα variants was assessed by Western Blot using polyclonal antibodies against PI4KIIIα (middle panel) or monoclonal antibodies against the HA-peptide (top panel), respectively. An arrowhead points to endogenous PI4KIIIα. (B) Replication of HCV was quantified by transfection of firefly luciferase reporter replicons into indicated cell lines. Replication efficiency is expressed as relative light units (RLU) normalized to 4 h after transfection to account for transfection efficiency. A replication-deficient deletion mutant in NS5B (ΔGDD) served as negative control. Data represent mean values and standard deviations of a representative experiment (n=3). Significances over shPI4K cells are shown for replication efficiencies 48 h after transfection. (C) Plasmids encoding for HCV NS3-5B were transfected into Huh7-Lunet T7 cell lines depleted of endogenous PI4KIIIα and stably expressing the indicated variants of PI4KIIIα (for details refer to the legend of Fig. 2). 24 h after transfection, cells were stained for PI4P and fluorescent signals were quantified by normalizing intensities of HCV-transfected cells to mock-transfected cells of each cell line. Standard deviations are indicated. At least 30 cells were quantified for each condition. (D) pTM constructs encoding for the indicated HA-tagged PI4KIIIα mutants were transfected into Huh7-Lunet T7 shPI4K cells. 24 h after transfection, cells were lysed and HA-tagged proteins were immunoprecipitated using HA-beads. Input and immunoprecipitated proteins were visualized by monoclonal antibodies directed against HA-peptide or β-actin, respectively. Shown are cropped images from the same Western Blot. (E) Immunoprecipitated HA-PI4KIIIα mutants were subjected to in vitro lipid kinase assays based on incorporation of [32P] into phosphatidylinositol substrates. Radioactive lipids were extracted and subjected to liquid scintillation counting. Depicted are counts per minute (cpm) obtained for the different PI4KIIIα mutants. (F) Huh7-Lunet shPI4K cells were co-transfected with plasmids encoding for the indicated HA-tagged PI4KIIIα variants and with constructs expressing HCV NS3 to NS5B. HCV NS5A or HA-tagged proteins were stained using monoclonal antibodies.
Depicted are mean values and standard deviations of Pearson correlation co-efficients of at least ten individual cells per condition. ***: p < 0.001, *: p < 0.05.

Figure 6: Importance of individual PI4KIIIα subdomains for HCV replication. (A) Schematic representation of the HA-tagged, C-terminal half of the PI4KIIIα protein used for further mapping of functional domains. Annotations of subdomains and motifs were adapted from Gehrmann et al. (13) or based on structure modelling (Δ1280-1430, Δ1430-1530, for details refer to Fig. 1 or 9, respectively). All numberings refer to GenBank accession number AB384703.

(B) Huh7-Lunet T7 were stably transduced with selectable lentiviral vectors expressing non-targeting shRNA (NT) or shRNA targeting endogenous PI4KIIIα (shPI4K) and encoding HA-tagged PI4KIIIα variants as indicated. Knockdown of endogenous PI4KIIIα and ectopic expression of HA-tagged PI4KIIIα variants was verified by Western Blot using polyclonal antibodies against PI4KIIIα (top panel) or monoclonal antibodies against the HA-peptide (middle panel), respectively. An arrowhead points to endogenous PI4KIIIα and an asterisk points to a PI4KIIIα variant which was not recognized by the polyclonal antiserum. (C) Replication of HCV was quantified by transfection of firefly luciferase reporter replicons into the indicated cell lines. Replication efficiency is expressed as relative light units (RLU) normalized to 4 h after transfection to normalize for transfection efficiency. A replication-deficient deletion mutant in NS5B (ΔGDD) served as negative control. Significances over shPI4K cells are shown for replication efficiencies 48 h after transfection. Data represent mean values and standard deviations of a representative experiment (n=3). ***: p < 0.001.

Figure 7: Enzymatic activity of PI4KIIIα Δ1-1101 proteins harboring additional subdomain deletions. (A) Plasmids encoding for HCV NS3-5B were transfected into Huh7-Lunet T7 cell
lines depleted of endogenous PI4KIIIα and stably expressing the indicated variants of PI4KIIIα upon lentiviral transduction (for details refer to the legend of Fig. 2). 24 h after transfection, cells were fixed and permeabilized with Digitonin. NS5A (red) or PI4P (green) were stained using monoclonal antibodies. Nuclei (blue) were stained using DAPI. (B) Fluorescence signals of PI4P from whole-cell z-stacks were quantified using ImageJ software. PI4P induction was quantified by normalizing intensities of HCV-transfected cells relative to mock-transfected cells of each cell line. Standard deviations are indicated. At least 30 cells were quantified for each condition. (C) Plasmids encoding for the indicated HA-tagged PI4KIIIα mutants were transfected into Huh7-Lunet T7 shPI4K cells. 24 h after transfection, cells were lysed and HA-tagged proteins were immunoprecipitated using HA-beads. PI4KIIIα and β-actin were visualized by western blot, using antibodies against the HA-peptide and β-actin, respectively, in the cell lysate (Input) and after IP. The ca. 46 kDa band in the IP fractions corresponds to the heavy chain of the antibodies used for IP. (D) Immunoprecipitated HA-PI4KIIIα mutants were subjected to in vitro lipid kinase assays based on incorporation of [32P] into phosphatidylinositol substrates. Radioactive lipids were extracted and subjected to liquid scintillation counting. Depicted are counts per minute (cpm) obtained for the different PI4KIIIα mutants after background subtraction. Note that recovery rates were reproducibly low for mutants ΔLKU and ΔPH, therefore low levels of residual lipid kinase activity formally cannot be excluded.

**Figure 8: Subcellular localization of PI4KIIIα domain deletion mutants in presence and absence of HCV NS proteins.** Huh7-Lunet shPI4K cells were transfected with plasmids encoding for the indicated HA-tagged PI4KIIIα variants (A, B, C) and in addition with constructs expressing HCV NS3 to NS5B (B, C). (A) Proteins were stained using monoclonal antibodies directed against PDI as marker of the ER (red) or HA-peptide (green), respectively. (B) HCV
NS5A or HA-tagged proteins were stained using monoclonal antibodies directed against NS5A (red) or HA-peptide (green), respectively. (C) 3D co-localization of NS5A and PI4KIIIα in experiments as shown in (B) was assessed by recording whole-cell z-stacks and analyzed using Huygens Essential software. Depicted are mean values and standard deviations of Pearson correlation co-efficients of at least ten individual cells per condition. *: p < 0.05.

**Figure 9: Homology modeling of the C-terminus of PI4KIIIα.** (A) Comparison of structural annotations of C-terminal PI4KIIIα domains by Gehrmann et al (13) and of results from homology modeling using PI3K as template (48). All numberings refer to accession number NP_477352.3. The region 1101-1535 cannot be modeled reliably, but secondary structure predictions suggest three helical subdomains (DI to DIII). (B) Secondary structure prediction of helical domain DI. Predicted helices are shown as waves and grey values correspond to the probability of helix formation. Regions that tend to disorder are marked with an underlined D. (C) Homology model of PI4KIIIα aa 1536-2102 colored according to the nomenclature established for the homologous PI3K. Blue: helical armadillo repeat domain (ARM), red and green: kinase domain, N-lobe (N-Cat) and C-lobe (C-Cat), respectively. The 1810-1840 region of PI4KIIIα (not modelled) would be inserted at the location marked by an asterisk. A location below the two lobes that is often occupied by regulatory and recruitment domains N-terminal to the homologous ARM domain in PI3K is highlighted as a yellow ellipse. A view rotated by 90° around the horizontal axis is given at the bottom. Note that aa 2103 in our PI4KIIIα variant is missing in all other Genbank entries and therefore was not included in the modelling. (D) Zoom on the interface between the N-lobe and C-lobe. The ATP pocket that is also the target of wortmannin is highlighted with a modeled ATP in gray spheres. The catalytic loop and activation

48
loop in the C-lobe are shown in cyan and magenta, respectively. The catalytic residue D1957 in the catalytic loop is displayed as sticks.
Colocalization with NS5A [Pearson co-efficient]

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pTM HA-PI4K ΔXXX + pTM NS3-5B JFH-1

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