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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 05 February 2013
Final form accepted: 17 July 2013
Published online: July 2013

Volume 18 (2013) pp 447-458
DOI: 10.2478/s11658-013-0099-7

Short communication

THE NTPASE/HELICASE DOMAIN OF HEPATITIS C VIRUS NONSTRUCTURAL PROTEIN 3 INHIBITS PROTEIN KINASE C INDEPENDENTLY OF ITS NTPASE ACTIVITY

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Abstract: Helicase motif VI is a short arginine-rich motif within the NTPase/helicase domain of the non-structural protein 3 (NS3) of the hepatitis C virus (HCV). We previously demonstrated that it reduces the catalytic activity and intracellular shuttling of protein kinase C (PKC). Thus, NS3-mediated PKC inhibition may be involved in HCV-associated hepatocellular carcinoma (HCC). In this study, we expand on our earlier results, which were obtained in experiments with short fragments of NS3, to show for the first time that the catalytically active, longer C-terminal NTPase/helicase of NS3 acts as a potent PKC inhibitor *in vitro*. PKC inhibition assays with the NTPase-inactive mutant

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Abbreviations used: ATP – adenosine triphosphate; CREB – cAMP response element-binding protein; ER – endoplasmic reticulum; HBV – hepatitis B virus; HCC – hepatocellular carcinoma; IFN-α – alpha-interferon; Km – Michaelis constant; Pi – inorganic phosphate; PKA – cAMP-dependent protein kinase; PKC – protein kinase C; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STAT – signal transducer and activator of transcription

NS3h-D1316A revealed a mixed type kinetic inhibition pattern. A broad range of 11 PKC isotypes was tested and all of the PKC isotypes were inhibited with IC_{50}-values in the low micromolar range. These findings were confirmed for the wild-type NTPase/helicase domain in a non-radiometric PKC inhibition assay with ATP regeneration to rule out any effect of ATP hydrolysis caused by its NTPase activity. PKCα was inhibited with a micromolar IC_{50} in this assay, which compares well with our result for NS3h-D1316A (IC_{50} = 0.7 μM). In summary, these results confirm that catalytically active NS3 NTPase/helicase can act in an analogous manner to shorter NS3 fragments as a pseudosubstrate inhibitor of PKC.

Key words: Hepatitis C virus (HCV), NS3 protein, Protein kinase C (PKC), PKC isotypes, Protein kinase inhibitors, Pseudosubstrate inhibition, Hepatocellular carcinoma (HCC)

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC) [1]. In previous studies, we showed that within its NTPase/helicase domain, NS3 contains an arginine-rich sequence motif consisting of residues 1487 to 1500 of HCV polyprotein, also known as helicase motif VI [2]. This motif strongly resembles the pseudosubstrate sequence within the auto-regulatory domain of protein kinase C (PKC) [3, 4] and it interacts with the active site of PKC, thereby inhibiting the catalytic activity and function of this enzyme [5, 6]. It has also been shown that fragments of HCV-NS3 that contain this motif can inhibit the catalytic activity of cAMP-dependent protein kinase (PKA) and slow down its cytoplasm-to-nucleus translocation [2, 7]. Similar interactions with protein kinases are well documented for several synthetic peptides that mimic the phosphorylation site sequences of the natural protein substrates or pseudosubstrate auto-regulatory regions of kinases [8, 9].

However, our previous studies on the interaction between HCV-NS3 and PKC were only conducted with smaller, catalytically inactive fragments of NS3. This may not have accurately reflected the situation *in vivo*. Furthermore, other groups have argued that the inhibition of PKA and possibly PKC by HCV-NS3 was rather due to the effect of NS3-4A-catalyzed ATP hydrolysis [10]. Here, we address the question whether the full-length NS3 NTPase/helicase domain can inhibit PKC independently of its NTPase activity.

In this study, we substantiate the results of our previous studies [5, 6] and overcome one of their substantiate limitations: the use of smaller non-physiological NS3 fragments. Moreover, we determine the PKC isotype specificity and the mode of inhibition for NS3 NTPase/helicase-mediated inhibition of PKC. We show that all PKC isotypes are inhibited by NS3 with IC_{50} values in the low micromolar range independently of the NS3 NTPase activity.

Our findings could be physiologically relevant because members of the PKC family play pivotal roles in cellular proliferation, apoptosis and cell survival, and there is a substantial amount of evidence linking disturbance of PKC to tumorigenesis [11]. It is plausible that NS3-mediated PKC inhibition may be involved in the molecular pathogenesis of HCV infection, particularly in the development of HCV-associated HCC.

MATERIALS AND METHODS

Reagents

Oligonucleotides were obtained from MWG Biotech AG (Germany). Restriction enzymes and T4 DNA-Ligase were obtained from New England Biolabs (USA) or Fermentas GmbH (Germany). [γ-^{32}P]-ATP (4500 Ci/mmol) was obtained from Amersham (USA). Highly purified protein kinase Cα (PKCα), in preparative amounts, was donated by Dr. S. Stabel (Max-Delbrück-Laboratory, Cologne, Germany). Rat brain PKC was bought from Calbiochem (USA). All other PKC isotypes were provided by Dr. C. Schächtele (ProQinase GmbH, Freiburg, Germany). Coding sequences for the PKC isotypes were taken from the following species: α, ε and η (mouse); βI, βII, θ, ι, μ and ζ (human); and γ and δ (rat).

Cloning, bacterial expression and purification of NS3h and NS3hD1316A proteins

The point mutation D1316A was introduced into pET21b-NS3HCV (provided by Dr. Choe [12]) via QuikChange mutagenesis (Stratagene, USA). Coding sequences for NS3h-D1316A and wt-NS3h were cloned into pET102/D-TOPO (Invitrogen, USA) to be expressed as N-terminal Thioredoxin and C-terminal *Strep*-Tag II fusions. The coding sequence for the *Strep*-Tag II (WSHPQFEK) followed by a stop codon was introduced to the 3' end of the coding sequence via the reverse primer. Recombinant proteins were expressed in *E. coli* BL21(DE3) as previously described [4], purified from sonicated bacterial pellets using *Strep*-Tactine sepharose affinity chromatography (IBA GmbH, Germany), and concentrated using Amicon Ultra centrifugal filter units (Millipore, USA).

ATPase assay

The reaction mixtures (20 μl) contained 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM 2-mercaptoethanol, 0.01% Triton X-100, 2 mM $MgCl_2$, 0.5 μCi [γ-^{32}P]ATP, ATP corresponding to the Km value, and the respective enzyme. These were incubated at 30ºC for 30 min and the reaction was terminated by incubation at 95ºC for 5 min. Apyrase (Adenosine 5'- triphosphatase; Sigma-Aldrich, USA) was used as a positive control. The formation of γ-^{32}P was assayed via thin layer chromatography of 1 μl of each reaction on POLYGRAM CEL 300 PEI plates (Macherey-Nagel, Germany) in 0.4 M KH_2PO_4 (pH 3.4). The dried plates were exposed to a PhosphorImager Screen (Amersham, USA).

Radiometric protein kinase C assay

The protein kinase C activity and kinetic parameters were determined as previously described [6].

Assay of PKC activity in the presence of an ATP regeneration system *in vitro*

The reaction mixtures (25 µl) containing 0.25 pmol PKCα (ProQinase GmbH, Freiburg, Germany), the PepTag substrate (from the PepTag Kit, Promega, USA), 3.5 mM ATP, 20 mM phosphocreatine (Sigma-Aldrich, USA), 1 unit of creatine phosphokinase (Sigma-Aldrich, USA), PKC Activator Solution and Peptide Protection Solution (Promega) in the volume recommended by the manufacturer, 20 mM HEPES (pH 7.4), 1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1 mM Dithiothreitol (DTT) and recombinant NS3h, as indicated, were incubated at 30ºC for 30 min. The reactions were stopped by placing the tubes on ice. The products were separated by 0.8% agarose gel electrophoresis in 0.5 M Tris/HCl (pH 8.0) and 3.5 M urea.

RESULTS

Expression and purification of NS3h and NS3h-D1316A

To investigate the *in vitro* interaction between the enzymatically active C-terminal NS3 NTPase/helicase and PKC isotypes, we used the previously well-characterized C-terminal NTPase/helicase (HCV polyprotein amino acids 1193-1658) of HCV-1 [12], expressed as N-terminal thioredoxin and C-terminal *Strep*-Tag II fusion protein. In addition to the wt-protein (NS3h), we generated an NTPase/helicase-deficient point mutant (NS3h-D1316A) [10, 13]. Recombinant proteins were purified by *Strep*-tag affinity chromatography as described in the Materials and Methods section.

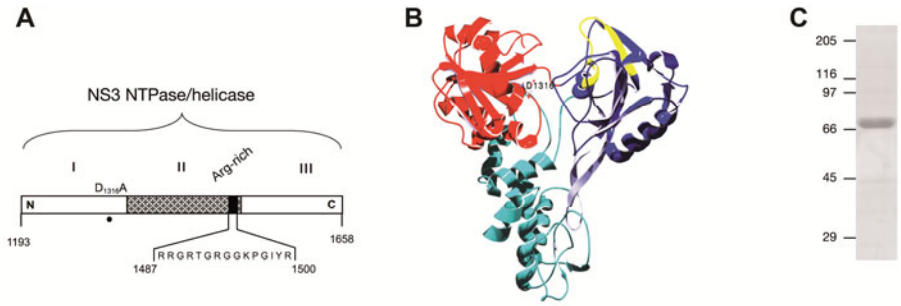

Fig. 1. The generation and purification of recombinant HCV NS3 polypeptides. The deployed recombinant proteins are shown as a schematic representation (A) and as a ribbon diagram (B), illustrating the positions of the NS3 NTPase/helicase subdomains and the arginine-rich stretch. The ribbon diagram was rendered using POV-Ray [15] based on the coordinates PDB 1HEI [16]. Subdomains 1, 2 and 3 are respectively red, blue and turquoise. C – The purity of the recombinant proteins was verified using SDS-PAGE. A representative 10% SDS PAGE gel of NS3h after staining with Coomassie Brilliant Blue is shown.

Fig. 1A and B depict the proteins used in this study. The obtained NS3 polypeptide preparations were confirmed as homogenous using SDS-PAGE (Fig. 1C). The purified NS3h protein exhibited an intrinsic ATPase activity that was strongly stimulated by the presence of poly(U) (Fig. 2A, B). These characteristics concur with those described in the literature [13, 14] and suggest a native conformation for NS3h. As expected, NS3h-D1316A did not exert any NTPase activity at the concentrations tested (Fig. 2A).

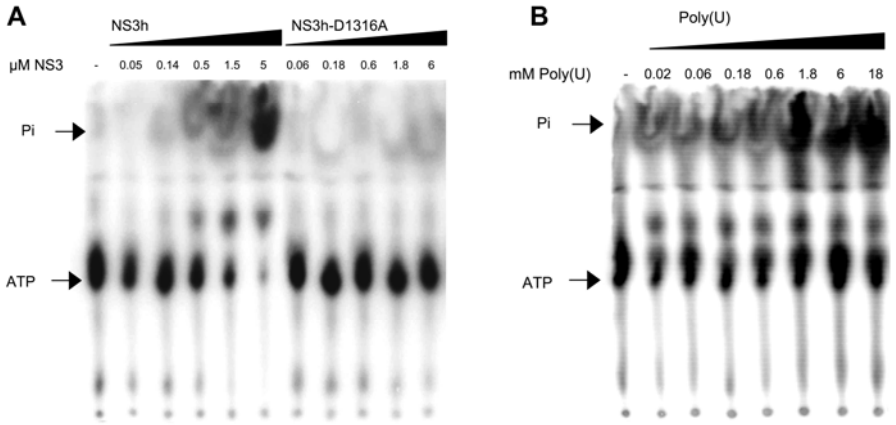

Fig. 2. ATPase activity of NS3h and NS3h-D1316A. Purified polypeptides were assessed in a thin layer chromatography-based ATPase assay using [γ-^{32}P]-ATP. A – Increasing amounts of NS3h and NS3hD1316A were assayed. B – The same assay was carried out at a constant concentration of NS3h (0.5 μM) in the presence of increasing amounts of Poly(U).

NS3h-D1316A and NS3h are potent inhibitors of PKC *in vitro*

The influence of NS3 constructs on PKC activity was determined *in vitro* by quantifying the phosphate incorporation into the PKC substrate histone HIIIS in the absence or presence of increasing concentrations of the recombinant HCV NS3 proteins. Since ATP to ADP conversion potentially interferes with PKC activity measurement in standard radiometric PKC assays by reducing the specific radioactivity of [γ-^{32}P]-ATP [10] we also tested the NTPase inactive mutant NS3h-D1316A for its capability to inhibit PKC. Fig. 3 shows the inhibition of PKCα by wt-NS3h and the NTPase-inactive mutant NS3h-D1316A in the radiometric PKC assay. The wt-protein (NS3h) inhibits the activity of PKCα with an apparent IC_{50} of 0.05 μM in this assay, which is in part due to ATP hydrolysis (see below). The IC_{50} for NS3h-D1316A was determined to be 0.7 μM. IC_{50} values for the inhibition of all 11 PKC isotypes by NS3h-D1316A were determined. All of the PKC isotypes were potently inhibited with IC_{50} values that fall within the low micromolar range (Table 1).

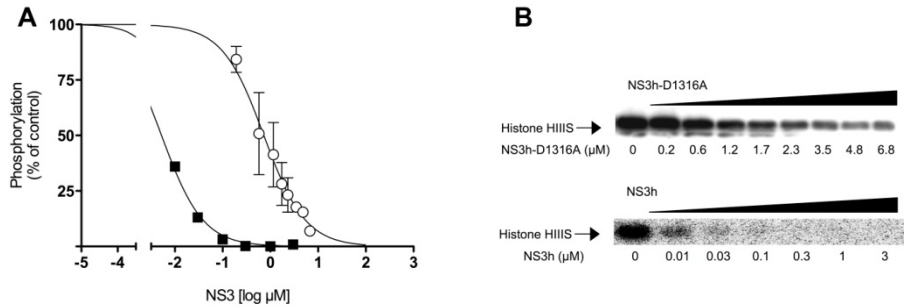

Fig. 3. Inhibition of the enzymatic activity of PKCα by NS3h and NS3hD1316A *in vitro*. A – Semi-logarithmic plot of the mean values for the inhibition of PKCα by NS3h (squares) and NS3h-D1316A (open circles) relative to the values from control experiments without NS3. The X-axis denotes the logarithm of the NS3 concentrations used in the PKC inhibition assay. PKC inhibition was determined by measuring phosphate incorporation into the PKC substrate histone HIIIS in presence of increasing concentrations of NS3 protein. The concentration of histone HIIIS was kept constant at 0.05 µg/µl. IC_{50} values were calculated by nonlinear regression. Error bars represent the standard error of the means. B – Corresponding exemplary autoradiograms showing the phosphorylated bands.

Table 1. Inhibition of PKC isotypes by NS3h-D1316A *in vitro*. IC_{50} values were determined in a radiometric PKC assay. Rat brain PKC is predominantly composed of the conventional PKC isotypes α, β1, β2 and γ.

Class	Isoform	IC_{50} (µM)
cPKC	α	0.7
	β1	3.4
	β2	0.8
	γ	0.3
	rat brain PKC	1.1
nPKC	δ	0.4
	ε	0.6
	η	0.9
	θ	1.8
aPKC	ι	0.3
	µ	0.3
	ζ	1.1

We also used a non-radiometric PKC assay that contained an ATP regeneration system (phosphocreatine and creatine phosphokinase [17]) to keep the ATP concentration constant during the course of the reaction in the presence of NS3h (Fig. 4). For this purpose, a commercial assay (PepTag, Promega) with a fluorescently labeled peptide PKC substrate was used, as previously described by Aoubala *et al.* [10]. As expected, PKC activity was completely abrogated in

the presence of 1.2 μM NS3h when the ATP regeneration system was absent (lane 1). When the full ATP regeneration system was included, the presence of 1.2 μM NS3h still caused a marked reduction in PKC activity (> 50%; lane 4), which compares well with the results for NS3h-D1316A and PKCα in the radiometric assay (IC_{50} = 0.7μM). These results demonstrate that NS3h inhibits the enzymatic activity of PKC independently of ATP hydrolysis in a similar range to NS3h-D1316A.

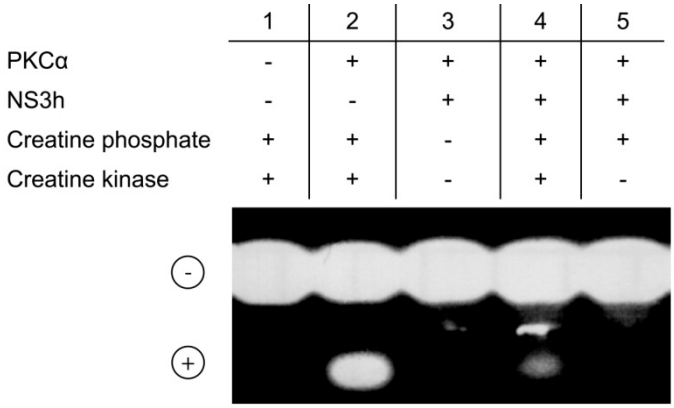

Fig. 4. Catalytically active NS3h inhibits PKCα when the concentration of ATP is maintained at a constant level. Results from a non-radioactive PKC assay based on the PepTag system (Promega) complemented with an ATP-regenerating system as described in the text. Lane 1: The full ATP regeneration system with neither PKC nor NS3h present, resulting in only one band corresponding to the non-phosphorylated peptide. Lane 2: The addition of PKCα results in the formation of the phosphorylated peptide with a net charge of -1. It migrates towards the positive pole, as evidenced by the appearance of the lower band. Lane 3 and 5: The presence of PKCα and catalytically active NS3h in full (lane 3) or partial (lane 5) absence of the ATP regeneration system results in total abolishment of PKC activity. Lane 4: The addition of catalytically active NS3h in presence of the full ATP regeneration system leads to partial inhibition of PKC activity, resulting in a weak lower band. NS3h was tested at a final concentration of 1.2 μM.

NS3h-D1316A acts as a mixed-type inhibitor of rat brain PKC

Finally, we aimed to determine the mode of inhibition for NS3 NTPase/helicase-mediated inhibition of PKC. We measured the inhibition of rat brain PKC by NS3h-D1316A at multiple concentrations of NS3 and the PKC substrate histone HIIIS. We then subjected the data to graphical analysis according to the methods described by Dixon and Cornish-Bowden [18, 19]. The resulting plots (Fig. 5) revealed that NS3h-D1316A acts as a mixed-type inhibitor of rat brain PKC. K_i (the dissociation constant for the enzyme-inhibitor complex) was 0.5 μM and K_i' (the dissociation constant for the enzyme-substrate-inhibitor complex) was 1.5 μM. The magnitude of K_i is considerably smaller than K_i', which suggests that the observed PKC inhibition is primarily due to specific interactions between NS3h-D1316A and the active sites of rat brain PKC.

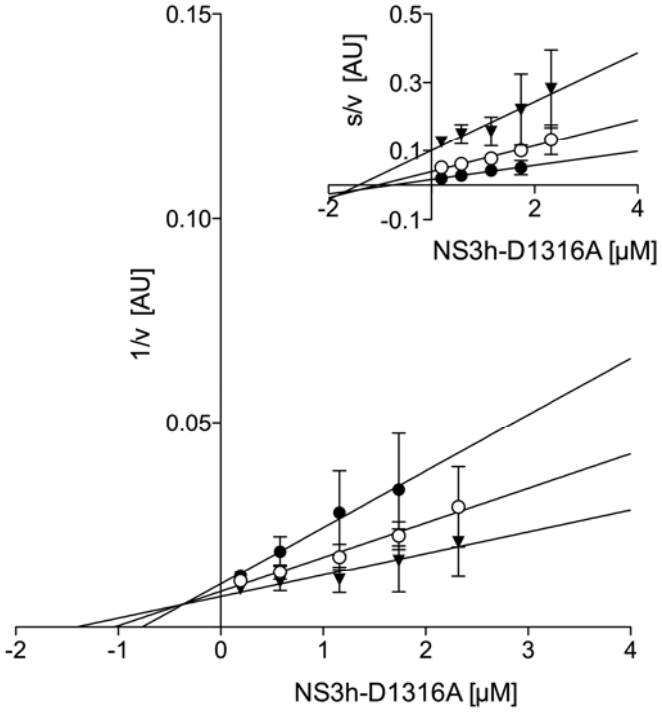

Fig. 5. PKC inhibition by NS3h-D1316A displays a mixed-type kinetic pattern. Data from radiometric PKC assays for the inhibition of rat brain PKC-catalyzed phosphorylation of histone HIIIS by NS3hD1316A at multiple substrate concentrations were plotted according to Dixon (main figure) and Cornish-Bowden (insertion). The intersections of the regression lines give K_i in the Dixon plot and K_i' in the Cornish-Bowden plot. The inhibitor constants K_i and K_i' were estimated to be 0.5 µM and 1.5 µM respectively. v – initial rate (velocity) of the reaction; s – substrate (histone HIIIS) concentration. Histone HIIIS concentrations were 0.03 µg/µl (open circles), 0.14 µg/µl (filled circles) and 0.34 µg/µl (triangles). The mean values from at least two independent experiments are shown. Error bars represent the standard error of the means.

DISCUSSION

The aim of this follow-up study was to resolve the question whether the full-length NTPase/helicase domain of HCV-NS3 can act similarly to its smaller fragments as an inhibitor of PKC *in vitro*. We also wished to expand on our previous findings with an additional analysis of the PKC isotype specificity of this interaction and the mode of action for NS3 NTPase/helicase-mediated inhibition of PKC. Most importantly, we can confirm that the enzymatic activity of PKC is indeed potently inhibited by the catalytically active C-terminal helicase portion of NS3 *in vitro*. The observed PKC inhibition is independent of the NTPase activity of NS3, as demonstrated by experiments with an NTPase-deficient point mutant. Additionally, a PKC activity assay containing an

ATP-regenerating system was employed to confirm these results for wild-type NS3-NTPase/helicase and consequently rule out the influence of the D1316A point mutation on the accessibility of the arginine-rich stretch.

Our data are in line with the results of our earlier studies [3, 5, 6, 20]. In those studies smaller NS3 fragments comprising the arginine-rich stretch [5, 6, 20], and notably the short peptide HCV-polyprotein (1496-1500) representing only the pseudosubstrate-like sequence [3], were all found to bind and inhibit PKC with IC_{50} values in the same order of magnitude. Together with our new finding that NS3h-D1316A acts as a mixed-type inhibitor of rat brain PKC, these novel results strongly suggest that NS3-NTPase/helicase-mediated inhibition of PKC is at least in part due to pseudosubstrate inhibition and follows a complex mechanism similar to the PKC inhibition by the shorter fragments analyzed in our earlier studies conducted [5, 20]. Further studies should focus on experimentally elucidating the exact mechanism of the complex interaction between PKC, its substrate and the inhibiting viral protein.

For most PKC isotypes, the IC_{50} value is within a similar range as determined earlier for the isolated subdomain 2 of NS3 NTPase/helicase [6]. PKC isotypes γ, δ, η and ζ are even more potently inhibited by NS3h-D1316A than by the isolated subdomain 2 with a roughly tenfold higher IC_{50}, suggesting additional interactions between these PKC isotypes, NS3 and/or the substrate histone HIIIS (as discussed in [20]).

At first glance, our results appear to be in conflict with the results of the study by Aoubala *et al.* [10] who did not observe NTPase-independent inhibition of protein kinase A (PKA) by full-length HCV NS3-4A. However, that study only explored the interactions of NS3 with PKA while our study is focused solely on PKC. The situation appears to be different for the two kinases. Interestingly, Aoubala *et al.* showed that although HCV NS3-4A does not inhibit PKA independently of its NTPase activity, it can still inhibit PKA-mediated CREB phosphorylation *in vivo*, probably through ATP depletion in the transiently transfected hepatoma cells that were used. Similar additional effects of the NS3 NTPase activity on PKC activity *in vivo* are conceivable and should be investigated in future studies.

These results raise the question why the hepatitis C virus might have evolved the ability to impair the functions of PKC. Interestingly, Fimia *et al.* could show that inhibition of conventional PKCs can prevent HCV replicon clearance mediated by alpha-interferon (IFN-α) [21]. It is intriguing to hypothesize that HCV may have evolved the capacity to disturb the activity of conventional PKCs (α, βI, βII and γ) as a mechanism to evade the IFN-mediated cellular response. Notably, Chen *et al.* showed in a recent study that hepatitis B virus (HBV) uses a similar evasion mechanism that involves impairment of IFN-α-induced STAT activation through inhibition of PKCδ by HBV polymerase [22]. There are several examples of viral proteins that interfere with PKC-mediated signal transduction to affect host cell functions [23-25].

We have demonstrated for the first time that the longer, catalytically active NS3-NTPase/helicase can act as a potent PKC inhibitor *in vitro*. All PKC isotypes are inhibited by NS3-NTPase/helicase to a varying degree, including the isotypes α and δ, for which inhibition or downregulation has been linked to carcinogenesis [11, 26].

Whether the determined inhibitory effects of NS3 NTPase/helicase on PKC are potent enough to be relevant *in vivo* will mainly depend on the exact local intracellular concentration of NS3 in infected cells, which is not known and difficult to assess. However, considering that HCV NS3 has been shown to localize to the endoplasmic reticulum (ER) or ER-like compartments when co-expressed with NS4A [27], high subcellular concentrations of NS3 in ER-compartments are conceivable. In future studies, it will be important to investigate the influence of HCV-NS3 on the activity of cellular PKC and related kinases *in vivo* in relevant cell culture systems harboring the viral protein, to clarify the physiological relevance of our findings.

Acknowlegements. This study was supported by a German Cancer Aid grant (PB, DKH 10-1654-B02). Julian Schulze zur Wiesch is supported by grants from the German Research Agency (SFB841-A6) and the DZIF (CL-15-2011). Philip Hartjen and Julian Schulze zur Wiesch were funded by the Werner Otto Stiftung. We would like to thank Dr. J. Choe (Korea Advanced Institute of Science and Technology) for providing the plasmid pET21b-NS3HCV, Dr. S. Stabel (Max-Delbrück-Laboratory, Cologne, Germany) for the generous gift of PKCα in preparative amounts, and Dr. C. Schächtele (ProQinase GmbH, Freiburg, Germany) for providing the full panel of PKC isotypes. This study is dedicated to our revered PhD mentor, Professor Peter Borowski, who passed away far too young, and before the completion of this study.

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