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Identification of cellular kinases responsible for Hepatitis C Virus NS5A hyperphosphorylation

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DOTTORATO DI RICERCA IN BIOLOGIA CELLULARE E MOLECOLARE
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Abstract

Hepatitis C virus (HCV) has been the subject of intensive studies for nearly two decades. Nevertheless, some aspect of the virus life cycle are still a mystery. The HCV Nonstructural protein 5A (NS5A) has been shown to be a modulator of cellular processes possibly required for the establishment of viral persistence. NS5A is heavily phosphorylated, and a switch between a basally phosphorylated form of NS5A (p56) and a hyperphosphorylated form of NS5A (p58) seems to play a pivotal role in regulating HCV replication.

Efficient replication of HCV subgenomic RNA in cell culture requires the introduction of adaptive mutations. Some of the most effective adaptive mutations occur at the serine residues that have been shown to be implicated in NS5A hyperphosphorylation and adaptive mutations at these sites result in a significant reduction of NS5A hyperphosphorylation (p58).

After screening of a panel of kinase inhibitors, we selected three compounds which inhibited NS5A phosphorylation in vitro, as well as the formation of NS5A p58 in cell culture. Cells transfected with the HCV wild type replicon sequence supported HCV RNA replication upon addition of any of the three compounds.

Thus, reduction of the formation of p58 below a certain threshold either by adaptive mutations or by inhibition of the NS5A–specific kinase(s) would enable HCV replication in cell culture.

Although large amounts of NS5A-p58 appear to inhibit HCV RNA replication, the complete inhibition of NS5A hyperphosphotylation by the kinase inhibitors we identified abolishes HCV replication of already adapted replicons indicating that a small quantity of p58 is required for replication.

Using kinase inhibitors that specifically inhibit the formation of NS5A-p58 in cells, we identified CK1 kinase family as a target.

NS5A-p58 increased upon overexpression of CK1α, CK1δ and CK1ε, whereas the RNA interference of only CK1α reduced NS5A hyperphosphorylation. Rescue of inhibition of NS5A-p58 was achieved by CK1α overexpression, and we demonstrated that the CK1α isoform is targeted by NS5A hyperphosphorylation inhibitors in living cells and that the down-regulation of NS5A attenuates HCV RNA replication.

Finally, we demonstrate here that NS5A is a direct substrate of CKI-α and phosphorylation of NS5A in vitro by CKI-α resulted in the production of two phosphorylated forms that resemble those products produced in cells.

In vitro kinase reactions performed with NS5A peptides show that S2204 is a preferred substrate residue for CKI-α after pre-phosphorylation of S2201.

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1. Introduction

1.1 Hepatitis C virus

Since the discovery of the hepatitis C virus (HCV) 15 years ago (*1*), it has been the subject of intense research and clinical investigations as its major role in human disease has emerged. It is estimated that 3% of the population is infected with this virus, and in 80 % of cases the virus establishes a chronic infection, resulting in fibrosis, cirrhosis and, increasingly, hepatocellular carcinoma (HCC).
Hepatitis C virus (HCV) is the only member of the genus Hepacivirus, within the family Flaviviridae. Major disease caused by the flaviviridae family includes Dengue fever, Japanese encephalitis, Yellow fever, Tick-borne encephalitis and the hepatitis C virus infection. Due to a high rate of spontaneous mutations, HCV exist as a heterogeneous group of viruses that have been classified into six genotypes (1–6) (fig 1).
Several methods are available to classify HCV genotypes. The most specific methods rely on PCR amplification of a region of the HCV genome followed by sequencing of the PCR product. The nucleotide sequence of the sample is compared to various established sequences to determine the degree of homology from which genotype is assigned (*2*).
Each of the six major genetic groups of HCV contains a series of more closely related subtypes (quasispecies) (Fig. 1) that typically differ from each other by 20–25 % in nucleotide sequences, compared with the >30 % divergence between genotypes (*3*). Some, such as genotypes 1a, 1b and 3a, have become distributed very widely as a result of transmission through blood transfusion and needle-sharing between infecting drug users (IDUs) over the past 30–70 years and now represent the vast majority of infections in Europe, U.S.A and Japan. These are the genotypes that are encountered most commonly in the clinical setting.
There is currently no vaccine or cure for HCV, but various treatments can reduce or stop virus replication.
Therapies for HCV currently approved by the United States Food and Drug Administration include interferon (IFN)-α and pegylated (polyethylene glycol conjugated) IFN-α, either alone or in combination with ribavirin (*4*). Sustained virological response (SVR) is defined by a reduction in serum viral RNA below the limits of detection (by reverse transcriptase polymerase chain reaction) for a 6-month period beyond completion of therapy. Optimal

therapy with pegylated IFN-α in combination with ribavirin is achieved in HCV genotypes 2 and 3, where an SVR of approximately 80% is possible in previously untreated patients; response rates with HCV type 1 genotypes are less remarkable, with an SVR of only approximately 40% after 1 year of combination therapy.
Research is ongoing to develop new and better medication.

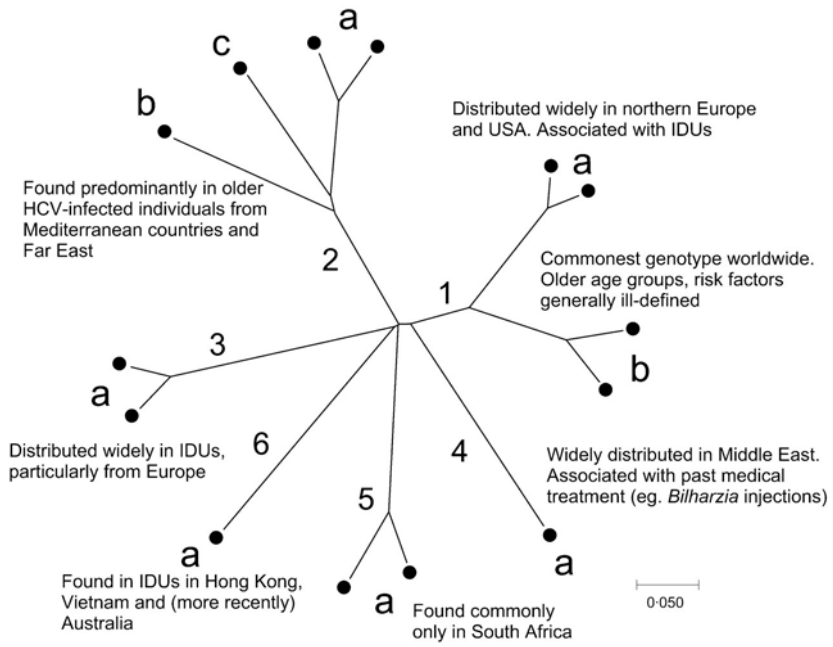

Fig 1

Evolutionary tree of the principal genotypes of HCV that are found in industrialized countries and their main epidemiological associations with specific risk groups.

1.2 Disease manifestation

The transmission of this virus occurs by several routes, most often by direct percutaneous exposure through sharing needles among injecting drug users or as a recipient of a contaminated blood. Sexual transmission of the virus appears to be very inefficient, as in maternal-infant transmission.
HCV accounts for about 20% of acute cases of hepatitis in the US. Yet acute infection is subclinical or mild in 65%-75% of patients. Roughly 20% of patients have jaundice and 10%-20% complain of malaise, anorexia, abdominal pain, or non-specific flu-like symptoms (center for disease control and prevention 1998). Fulminant hepatic failure is reportedly rare.
The incubation period averages about 50 days, but may be as short as 15 days and as long as 150 days.
Following acute infection it is estimated that only 15-20% of patients will clear the virus. The specific mechanisms underlying viral persistence are not known.
Infection tends to be insidious and sub clinical, most often discovered during the evaluation of abnormal liver function tests obtained on routine exams. The spectrum of severity of the liver disease associated with HCV varies widely from non-specific, minimal inflammatory changes to cirrhosis and hepatocellular carcinoma (*5*). The rate of progression of chronic hepatitis C is also variable, depending on many cofactors, mostly host-related, such as age, gender, alcohol consumption, overweightness and coinfections (*6*).

1.3 HCV Life Cycle

While little is known about the exact natural processes of hepatitis C, like other viruses, it must complete five key steps to carry out its life cycle:

1) The virus locates and attaches itself to a liver cell. Hepatitis C uses particular proteins present on its protective lipid coat to attach to a receptor site.
2) Fusion of the viral and cellular membranes, presumably triggered by the low pH of the endocytic compartment, leads to the release of a single-stranded (ss), positive-sense RNA genome into the cytoplasm of a newly infected cell.
3) The HCV RNA is then translated by the host ribosomes and begins the production of materials necessary for viral replication. Because hepatitis C stores its information in a "sense" strand of RNA, the

viral RNA itself can be directly read by the host cell's ribosomes, functioning like the normal mRNA present in the cell.

4) The non-structural proteins (NS2-NS5b) are next assembled and localized within the liver cell to form a replication complex which produces multiple copies of the HCV RNA genome.
5) These RNA copies are then able to re-enter the life cycle, producing more HCV proteins. Eventually, the HCV structural proteins (C, E1 and E2) along with copies of the HCV RNA are packaged as infectious virus particles, released from the liver cell, and are able to infect new cells.

1.4 Genomic organization of hepatitis C virus

HCV is a positive sense single stranded RNA virus with a genome containing approximately 9500 nucleotides. It has a single open reading frame that encodes a large polyprotein of about 3000 amino acids (fig 2). The genomic organization of HCV consists of a 5' untranslated region (5' UTR) of about 340 base pairs and downstream to the 5' UTR there are regions that encode the following proteins: the structural proteins "core", envelope 1, envelope 2, p7, and the non-structural protein 2, non-structural protein 3 (NS3), non-structural protein 4A and 4B, and non-structural protein 5A and 5B (NS5A and NS5B) (Fig.2). Downstream to this coding region there is another untranslated region of approximately 200 nucleotides (3' UTR).

The 5' UTR is highly conserved and therefore is the target for diagnostic assays. It has a complex secondary structure and contains the putative internal ribosome entry site (IRES), with an RNA sequence that directs the ribosomes to initiate translation (*7*).

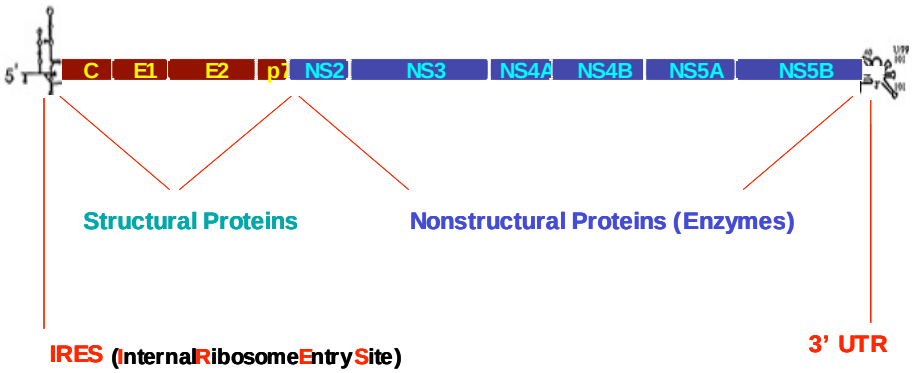

Fig.2
Structure of the HCV genome. The genome of HCV consists of a single open reading frame encoding a polyprotein of approximately 3010 aa, flanked by UTRs. The 5' UTR contains an IRES. In addition, both UTRs are highly structured (predicted structures are depicted) and are involved in initiation of RNA synthesis.

1.4.1 Polyprotein processing

The polyprotein is cleaved into 10 polypeptides by cellular and viral proteinases (fig.3).

Cleavages within the structural region and at the p7/NS2 junction are thought to be mediated by host cell signal peptidase(s), which are located in the lumen of the endoplasmic reticulum (ER) and cleave behind stretches of hydrophobic amino acids. Processing of the NS region is mediated by two overlapping virus-specific proteases. The NS2-NS3 zinc-dependent autoproteinase is essential for cleavage at the NS2/3 site. The NS3 serine protease utilizes NS4A as a cofactor to efficiently cleave the polyprotein at all sites downstream of the NS3 carboxy terminus, i.e., at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites (*8*). In addition, the expression of another HCV protein with yet unknown function has recently been described, the so-called F-protein, which is generated by ribosomal frameshifting (*9*).

1.4.2 The structural proteins

The HCV core is a highly basic protein of 21 kDa and constitutes the major component of the nucleocapsid; Core has RNA binding capacity and appears to be associated with the HCV genome. It has been suggested that this protein induces hepatic steatosis in transgenic mice and is an important factor in the development of hepatocellular carcinoma in patients with chronic HCV infection (*10*).
HCV envelope 1 and envelope 2 proteins are of about 37 and 61 kDa and they interact with each other and form a heterodimer.
The complex E1–E2 complex is expressed on the surface of the HCV particle (*11*).
The envelope 1 region is used for genotyping for clinical purposes. The first 81 nucleotides of the envelope 2 protein encode the hypervariable region 1 (HVR1) of HCV (*12*). HVR1 appears to induce the production of neutralizing antibodies and could function as a decoy to help the virus escape the immune system. The complex E1-E2 is the obvious candidate ligand for cellular receptors. Several candidate receptors for HCV have recently been put to the fore. Given the physical association of HCV particles with low- or very-low-density lipoproteins (LDL or VLDL) in serum, the LDL receptor has been proposed as a candidate receptor for HCV (*13*). The LDL receptor has been shown to mediate HCV internalization by binding to virion-associated LDL particles (*13*), but there are no experimental data to indicate that these interactions lead to productive infection. Using a soluble form of E2 as a probe, researchers have identified tetraspanin CD81 (*14*), the scavenger receptor class B type I (SR-BI) (*15*), and the mannose binding lectins DC-SIGN and L-SIGN (*16, 17*) as other putative HCV receptors. However, simple virus binding to a surface molecule does not necessarily mean that it is a receptor, and it is probable that several molecules combine to form a receptor complex (*14, 18, 19*).
The p7 protein was recently shown to belong to the viroporin family and to form an ion channel, therefore potentially playing an important role in viral particle release and maturation (*20*).

1.4.3 The non structural protein

NS2 is a nonglycosylated transmembrane protein of 23-kDa anchored to the endoplasmic reticulum (ER) (*21*) (*22*). NS2 in association with NS3 (NS2/NS3 protease) is the first activated viral protease within the HCV polypeptide responsible for the maturation of the remaining NS proteins (*23*). This NS2/NS3 autoprotease is essential for highly productive in vivo replication (*24*).
The N-terminal one-third of NS3 is a serine proteinase which, when associated with NS4A, is responsible for downstream cleavage of the HCV polyprotein (*25*). The 3D structure of NS3 serine proteinase, both free and complexed with NS4A, was recently determined (*25*), opening the way to the development of specific inhibitors. The C-terminal two-thirds of NS3 possesses helicase and NTPase functions. The 3D structure of this domain has also been determined, both free and complexed with DNA (*25*), again opening the way to the discovery of specific inhibitors. NS4B is an integral membrane protein that appears to play a role in the formation of a cellular membranousus web important for the assembly of the replication complex (*25*).
NS5B is the key enzyme of the replicase that promotes synthesis of new RNA genomes is the NS5B RNA-dependent RNA polymerase (RdRp). The NS5B RNA-dependent RNA polymerase (RdRp) is a tail-anchored protein, characterized by a transmembrane domain at the C-terminus of the protein responsible for posttranslational membrane targeting (*26, 27*). The structural organization of NS5B is a typical 'right hand' polymerase shape with finger, palm, and thumb subdomains surrounding a completely encircled active site (*28*). Replication proceeds *via* synthesis of a complementary minus-strand RNA using the genome as a template and the subsequent synthesis of genomic plus-strand RNA from this minus-strand RNA intermediate. As central component of the HCV replicase, NS5B has emerged as a major target for antiviral intervention (*29*).

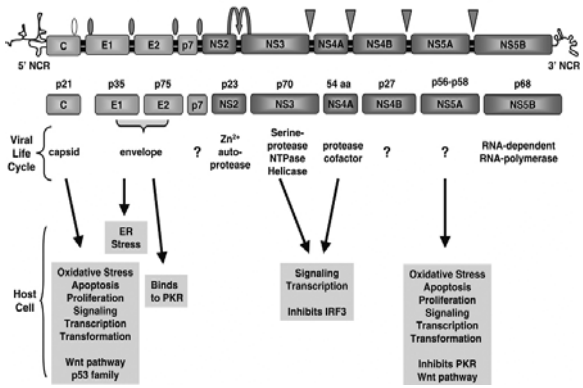

Fig3
Structural and Non-structural gene polyprotein processing.
The polyprotein precursor is processed into structural and non-structural proteins by cellular and viral proteases. Solid rods denote cleavage sites of the endoplasmic reticulum signal peptidase. The open rod indicates the additional C-terminal processing of the core protein by signal peptide peptidase. Arrow heads indicate cleavages by HCV NS2-3 and NS3 proteases The role of the NS2–3 proteinase appears to be limited to autoproteolytic cleavage of the NS2–3 junction in cis, between residues Leu-1026 and Ala 1027 at the sequence Gly-Trp-Arg-Leu-Leu-↓-Ala-Pro-Ile.

1.5 NS5A

HCV NS5A is a 447-amino-acid phosphoprotein, mainly phosphorylated on serine and to a much lesser extent, on threonine residues.

A major limitation in our understanding of NS5A is the paucity of structural information.

1.5.1 Structure and characterization of the NS5A protein

A number of structural features of the protein have, however, been derived experimentally.

N-terminal amphipathic α-helix mediates membrane association of NS5A (*30-32*). This helix exhibits a hydrophobic, tryptophan-rich side embedded in the cytosolic leaflet of the membrane bilayer, whereas the polar, charged side is exposed to the cytosol. Thus, NS5A is a monotopic protein with an in-plane amphipathic α-helix as membrane anchor. Recent structure-function

analyses showed that this helix displays fully conserved polar residues at the membrane surface, which define a platform probably involved in specific protein-protein interactions essential for the formation of a functional HCV replication complex (*30*). The identification and structural characterization of the in-plane membrane anchor of NS5A facilitated the rational design and expression of a recombinant soluble form of NS5A.
Limited proteolysis of recombinant NS5A has recently allowed specification of a domain organization of this protein predicted by comparative sequence analyses. domain 1 (amino acids 36–213), domain 2 (amino acids 250–342), and domain 3 (amino acids 356–447) (*33*) (Fig. 4).
The region of NS5A proposed to constitute **domain I** represents the most conserved portion of this protein among the HCV genotypes.
Interactions with karyopherin Δ3 (*34*), apolipoprotein A1 (*35*), phosphoinositide 3-kinase p85 subunit (*36*), $hTAF_{II}32$ (*37*), homeodomain protein PTX1 (*38*), and the La autoantigen (*39*) have been mapped to various portions of domain I. The proposed proximity of domain I to the cellular membrane is compatible with the properties of a number of these domain I interacting proteins that are either membrane associated or involved in membrane trafficking. Many of these interactions are intriguing, but are of unknown relevance to HCV biology. The goal of future research will be to identify a function for NS5A, and it seems likely that the answer would lie in the conserved, well-ordered region of the protein such as domain I.

Tellinghuisen et al. (*40*) recently reported the x-ray structure at 2.5 Å resolution of the relatively highly conserved domain I of NS5A (fig.5), which immediately follows the membrane-anchoring amphipathic α-helix. Based on the 3-dimensional structure, they divided domain I into an N-terminal subdomain IA and a C-terminal subdomain IB (Fig. 5A). Subdomain IA consists of an N-terminal extended loop lying adjacent to a 3-stranded anti-parallel β-sheet (B1-B3) with an α-helix, designated as H2 to allow numbering of the N-terminal membrane-anchoring amphipathic α-helix as H1, at the C-terminus of the third β-strand. These elements constitute the structural scaffold for a 4-cysteine zinc coordination site previously predicted based on biochemical studies by the same authors (*33*). This site is composed of the 4 fully conserved cysteine residues Cys 39, Cys 57, Cys 59, and Cys 80 that have previously been shown to be absolutely required for HCV RNA replication (*33*) (Fig. 5B). Based on the location of the zinc coordination site and their previous biochemical data, the authors suggest that this essential zinc has a structural rather than a functional role in NS5A.

Subdomain IB consists of 2 anti-parallel β-sheets (B4-B7 and B8-B9) surrounded by extensive random coil structures. No similar structures have thus far been reported, indicating that this protein represents a novel fold. A surprising finding was the presence of a disulfide bond near the C-terminus of subdomain IB, connecting the conserved cysteine residues Cys 142 and Cys 190 (Fig. 5A). Previous site-directed mutagenesis indicated that the disulfide bond formed between Cys 142 and Cys 190 is not essential for HCV RNA replication (*33*).

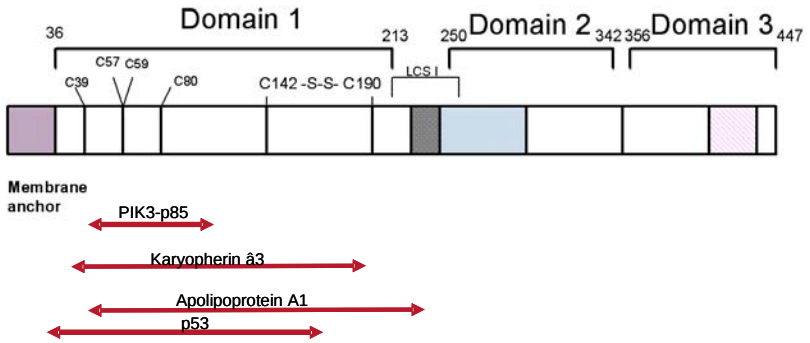

Fig4

Overview of the HCV NS5A protein. NS5A is drawn to scale as a box. Amino acid positions relate to the HCV Con1 sequence (genotype 1b; GenBank accession number AJ238799; add 1972 amino acids to obtain positions relative to the HCV polyprotein). The domain organization proposed by Tellinghuisen et al. (*33*) is shown. Domains I-III are connected by low-complexity sequences (LCS) I and II. Cysteine residues 39, 57, 59, and 80 coordinate one zinc atom per NS5A protein. Cysteine residues 142 and 190 form a disulfide bond. The N-terminal amphipathic α-helix, which mediates in-plane membrane association of NS5A, is highlighted by a gray-pink box.

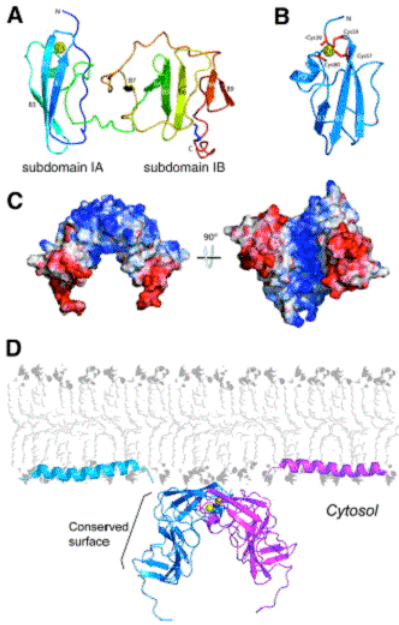

Fig 5
Structure of domain 1 of NS5A
Ribbon diagram of the structure of domain I (amino acids 36-198). The polypeptide chain is colored from the N terminus (blue) to the C terminus (red). The coordinated zinc atom is shown in yellow. The C-terminal disulfide bond is shown in blue. (B) A view of subdomain IA (amino acids 36-100), highlighting the zinc-binding motif. The zinc atom (yellow) is coordinated by four cysteine residues (red). (C) Surface-potential plots of the domain I dimer. The dimer creates a relatively flat, basic surface near the N terminus and a large groove with a basic bottom thought to bind single- or double-stranded RNA between the two subdomain IB regions. (D) The dimeric form of domain I modeled in relation to the membrane. The polar heads and the aliphatic tails of the phospholipids bilayer are gray and light gray, respectively. The two NS5A domain I monomers are colored in blue and magenta. Also shown in blue and magenta are the N-terminal amphipathic membrane anchors of NS5A lying flat in the plane of the membrane. Zinc atoms coordinated by domain I are shown as yellow spheres. This orientation of domain I places the largely basic surface toward the phospholipid head groups of the membrane and positions the large "claw" or groove of the NS5A dimer away from the membrane, where it may interact with RNA. Figure panels were provided by Dr. Timothy L. Tellinghuisen, The Rockefeller University, New York.

The proposed **domain 2** region of NS5A is of lower sequence conservation among HCV genotypes than domain I**.**
This region contains a number of predicted secondary structural elements, primarily α-helical, and it was suggested that it has a defined structure, but recently, Yu Liang et al. (*41*) performed gel-filtration study, CD, 1D 1H NMR and 2D 1H -15N HSQC analyses to characterize the biochemical

behavior of the NS5A-D2 (domain 2) and define its structural basis, and the results suggest that NS5A-D2 appears to contain high amount of flexible and unstructured regions with potentially small amount of α-helices. It was suggests that the non-structured regions of proteins are essential for protein functions.

The domain 2 (fig 6) appears to participate in several important biological regulations.

It was shown that the NS5A interacts with NS5B both *in vitro* and in transiently transfected cells. This binding required residues 105–162 (domain 1) and 277–334 (domain 2) of NS5A (*42*) and four discontinuous regions of NS5B. It was shown that deletions within NS5A that abrogated the interaction with NS5B also rendered the subgenomic replicon non-functional (*43*), whereas a deletion that had no effect on NS5B binding was replication-competent. The authors conclude that NS5A–NS5B interactions are necessary for HCV RNA replication; however, an alternative interpretation – that these deletions perturb the structure of NS5A, thereby abrogating another function associated with genomic replication – should also be considered.

The domain 2 also contains the Interferon (IFN) sensitivity-determining region (ISDR, amino acids 237-276) and the PKR binding domain (amino acid 237-309).

NS5A was first linked to IFN-responsiveness in patients by molecular epidemiological studies (*44*) that identified a stretch of 40 aa in the centre of NS5A conserved in IFN-resistant HCV isolates (Fig. 6). HCV variants with mutations within this region appeared to be more sensitive to therapy, suggesting that NS5A played a role in conferring IFN resistance, thus this region was termed the IFN sensitivity determining region (ISDR). Other groups confirmed these data, showing that expression of NS5A from IFN-resistant HCV1b isolates rendered cells partially resistant to the effects of IFN and allowed growth of either vesicular stomatitis virus (VSV) (*45*) or EMCV (*46*). NS5A isolates from genotype 1a or 2a IFN-responders or 1b ISDR-deleted mutants did not inhibit IFN activity in these assays. However, two studies (*47*, *48*) failed to find a correlation between ISDR sequence and ability to inhibit IFN activity, and subsequently it has been suggested that sequences towards the C terminus of NS5A, outwith the ISDR, are required to inhibit IFN activity (*49*).

Biochemical data have shown that NS5A bound to and inactivated PKR (*50*), an IFN-induced gene product that is activated by binding to dsRNA, commonly produced during RNA virus genome replication. PKR

phosphorylates the translation initiation factor eIF-2α, shutting down protein translation (*51*). The interaction of NS5A with PKR required the ISDR and an additional 26 residues C-terminal to the ISDR (fig 6) (*52*). Within PKR, the binding site was identified as the dimerization domain. Binding of NS5A to PKR resulted in inhibition of both PKR autophosphorylation and phosphorylation of an exogenous substrate. However, more recently, conflicting results have been presented. (*53, 54*) and were unable to observe any effect of NS5A (from either IFN-responders or non-responders) on the activity of PKR in either Huh7 or HeLa cells, although Podevin *et al*. did show that all isolates of NS5A inhibited IFN activity (using both EMCV and VSV). Furthermore, Podevin *et al*. were also unable to detect an NS5A–PKR interaction either by co-immunoprecipitation or by co-immunofluorescence.

The region of NS5A hypothesized to comprise **domain 3** is the most variable among HCV genotypes. This region contains large regions predicted to be random coil structures by various secondary structure prediction programs. The ability to generate functional NS5A proteins containing in-frame fusions with other proteins (*i.e.* green fluorescent protein) within domain 3 in the replicon system highlights the sequence flexibility of this region (*55*). This region does contain several regions of sequence that are absolutely conserved among all HCV sequences suggesting some selective evolutionary pressure exists for maintenance of this region. A careful evaluation of the regions of domain III required for HCV RNA replication is warranted.

One area of particular interest in evaluating the domain model of NS5A is the proposed surface exposure of the interdomain connector sequences (LCS I and LCS II) in relation to the HCV literature. It is important to note that designating a sequence "low complexity" does not imply that this sequence has no function. Indeed, examination of sequence alignments of various genotypes of HCV shows these low complexity sequence blocks tend to have considerable sequence conservation, thereby suggesting some importance to NS5A function and HCV biology. LCS I overlaps the region where numerous NS5A cell culture replicon adaptive mutations, some capable of increasing RNA replication 10,000-fold, have been described (*56*). It is enticing to hypothesize that the exposed residues comprising the LCS I region are a loop region connecting domain I to domain II, and that this loop region is involved in interaction with viral and cellular components of the replicase in a regulatory fashion or modulating contacts between the domains of NS5A. Similarly to LCS I, the sequence at LCS II is conserved among HCV isolates, suggesting a selective pressure for the maintenance of this region. Several publications suggest this proline-rich region of NS5A

contains a class II polyproline motif like sequence (amino acids 350–356) involved in interaction with Grb2 and downstream cellular signaling cascades (*57*) (*58*). This sequence has also been shown to interact with the cellular protein amphiphysin II (*59*). The demonstration LCS I and LCS II are exposed on the surface of NS5A, and available for potential interactions with viral and cellular factors, is an important observation. These ideas clearly need to be evaluated critically, but the data provide some intriguing hints at NS5A function and interactions.

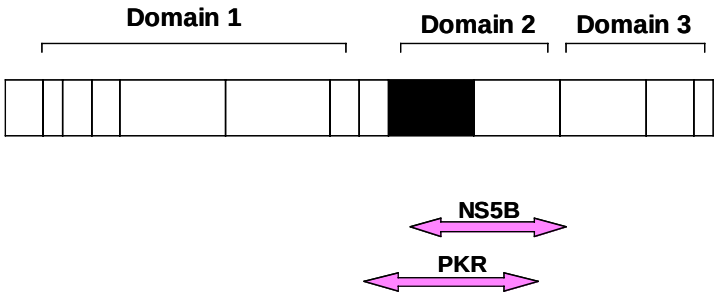

Fig.6

Overview of domain 2 futures:

The domain 2 contains the Interferon (IFN) sensitivity-determining region (ISDR, amino acids 237-276) and the PKR binding domain (amino acid 237-309) and the binding residues (277–334) of for NS5B interaction.

1.5.2 NS5A phosphorylation

Two phosphorylated forms of NS5A, termed p56 and p58, can be distinguished by their electrophoretic mobility. The p56 form of NS5A is a basally phosphorylated protein, modified by phosphorylation in the centre and near the C terminus. Although the sites of basal phosphorylation have not been identified, data suggest that it is mediated by members of the CMGC kinase. Inhibitor studies failed to define the kinase(s) involved (*60*); however, other studies showed that NS5A stably associated with both CKII (*61*) and cAMP-dependent protein kinase (*62*), both of which were able to phosphorylate a GST–NS5A fusion protein *in vitro*.

Coito *et al*. (*63*) screened the yeast 'kinome' for the ability to phosphorylate baculovirus-expressed NS5A *in vitro* and identified eight candidate kinases. NS5A was phosphorylated by the human homologues of these kinases,

including CKI and II, MAPK kinases MEK1, MKK6 and MKK7β1 and, lastly, Akt and p70S6K. Interestingly, two of these (MEK1 and Akt) are indirect targets for regulation by NS5A itself.
The p58 protein presents the hyperphosphorylated form of p56 (*64, 65*). The centrally located serine residues Ser 225, Ser 229, and Ser 232 (corresponding to Ser 2197, Ser 2201, and Ser 2204 of the HCV polyprotein) are important for NS5A hyperphosphorylation (Fig.6). However, whether these residues are actually phosphorylated or affect the NS5A phosphorylation state indirectly is unknown, because only few phosphoacceptor sites have been mapped experimentally (*66, 67*). A number of kinases capable of phosphorylating NS5A have been identified. However, which cellular kinase generates the different phosphoforms of NS5A during the viral life cycle was unknown before our work.
Several groups demonstrated that Hyperphosphorylation is dependent on the presence of other non-structural proteins. NS4A (expressed in trans) was required for the production of the p58 NS5A form of an HCV1b isolate (HCV-J) (*64, 68*). Furthermore, residues 162–166 of NS5A were required for NS4A-dependent phosphorylation. Interestingly, deletion of the N-terminal 162 residues of NS5A resulted in NS4A-independent hyperphosphorylation. The reason for this observation is unclear. Perhaps binding of NS4A might induce a conformational change in NS5A, facilitating hyperphosphorylation? Deletion of the N terminus of NS5A could thus alter the overall conformation of the protein allowing access by the kinase(s). Liu et al. (*69*) using another HCV1b isolate (HCV-BK), demonstrated a requirement for NS2 generated by autoproteolysis from the NS2–3 precursor. Two groups (*70, 71*) showed that hyperphosphorylation of an HCV1b (Con1) NS5A required its expression as part of a continuous polyprotein with NS3, NS4A and NS4B, and required the protease activity of NS3.

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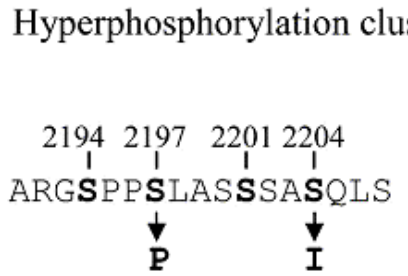


Fig.6
Central hyperphosphorylation cluster. Bolded serines were identified as sites of hyperphosphorylation – Ser2194 (*67*) or Ser2197, Ser2201 and Ser2204 (*72*). Loss of the hyperphosphorylation site Ser2197 (mutation to Pro) (*73*) or Ser2204 (mutation to Ile) (*56*) resulted in the indicated increases in the efficiency of colony formation by the subgenomic replicon.

1.5.3 NS5A and the virus replication

Investigation of the HCV life cycle has been limited by the lack of an efficient cell culture system permissive for HCV infection and replication. Nevertheless, considerable progress has been made using heterologous expression systems (*74*) ((*75-77*), functional cDNA clones, and, more recently, the replicon system (*78*) and the infection system (*79*). The replicon system has allowed, for the first time, to study efficient and genuine HCV RNA replication in Huh-7 human hepatoma cells in vitro. Replicons consist of bicistronic RNA transcripts in which the HCV IRES drives translation of neomycin phosphotransferase; a second IRES [from encephalomyocarditis virus (EMCV)] drives translation of NS3–NS5B. I*n vitro* transcripts are transfected into the human hepatoma cell line Huh7 and stable cell clones selected with G418. These cells then harbour a cytoplasmic, autonomously replicating RNA and express large amounts of NS3–NS5B**.** Moreover, the replicon system has clearly shown that the HCV nonstructural proteins 3–5B form an independent module sufficient for autonomous HCV RNA replication.

1.5.3.1 NS5A is part of a multi-protein, membrane-bound replication complex

The replicon system has shown that the replication complex contains NS5A. Together with the other non-structural proteins, NS5A co-localized with

viral RNA in a cytoplasmic membrane structure termed the 'membranous web'(*80*) (fig.7).
Formation of a membrane-associated replication complex, composed of viral proteins, replicating RNA, and altered cellular membranes, is a hallmark of all plus-strand RNA viruses investigated thus far (*80*). The role of membranes in viral RNA synthesis is not well understood. It may include the physical support and organization of the RNA replication complex, the compartmentalization and local concentration of viral products, tethering of the viral RNA during unwinding, provision of lipid constituents important for replication, and protection of the viral RNA from double-strand RNA-mediated host defenses or RNA interference.
The evidence for a critical role of NS5A in the replication of the virus came from experiments in which the amphipathic membrane-targeting helix was mutated in the context of the replicon (*30*). Introduction of three helix-disrupting mutations completely abrogated the ability of the replicon to establish G418-resistant colonies, implying that NS5A membrane association is an indispensable event during HCV RNA replication.

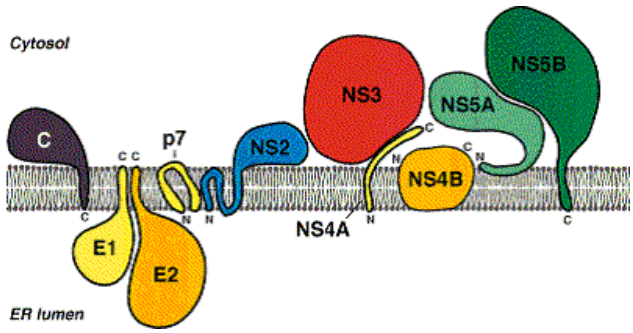


Fig7
HCV replication complex. Membrane association of HCV proteins. Note that the topologies of NS2, NS4A and NS4B are currently under investigation and are only schematically illustrated.

1.5.3.2 Culture adaptation of the replicon provides further evidence of a role for NS5A in RNA replication

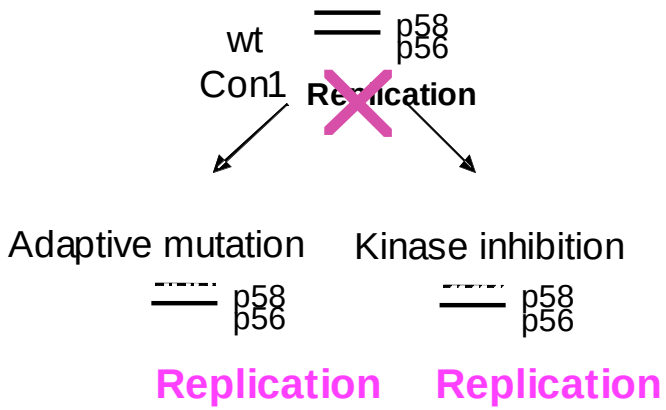
Replication of the replicon dramatically increases after the occurrence of adaptive mutations.

Krieger *et al*. (*73*) identified a single amino-acid substitution in NS5A (Ser2197Pro) that enhanced colony formation 1000-fold. Blight *et al*.(*56*) identified a cluster of mutations in NS5A between residues 2177 and 2204, as well as a deletion of 47 aa (2207–2254), that each stimulated colony formation. The most effective of these (20 000-fold stimulation) was Ser2204Ile. The most interesting observation from these studies is that both Ser2197 and Ser2204 were shown previously to be required for hyperphosphorylation (Fig. 6); indeed, reduced levels of the p58 form of NS5A were observed in cells harbouring replicons with mutations at these residues (*56*). The implication of these results is that NS5A hyperphosphorylation is dispensable for viral RNA replication and, together with the observations that hyperphosphorylated NS5A is not a component of the membrane-bound RNA replication complex (*81, 82*), these data suggest that this form of NS5A may play a distinct role in the virus life cycle, perhaps during particle assembly. This suggestion is further supported by the observation that, after introduction into a chimpanzee of an RNA transcript derived from an infectious clone containing the Ser2197Pro mutation, within 7 days the virus reverted to the parental sequence at that position (*83*).

2. Results

2.1 In vitro inhibition of NS5A phosphorylation by protein kinase inhibitors.

Since the identification of adaptive mutations in the HCV subgenomic replicon, it has been noted by several groups that the hyperphosphorylated form of NS5A, p58, seems not to be necessary for replication in cell culture. The starting hypothesis of our work was that high amounts of the NS5A hyperphosphorylated form, p58, inhibit replication and that reduction of p58 by inhibition of specific kinase(s) below a certain threshold enables HCV replication (scheme 1).



Scheme 1: Our hypotesis
It has been observed that adaptive mutations which reduce NS5A hyperphosphorylation activate replication.
We want to demonstrate that kinase inhibitors, which reduce NS5A hyperphosphorylation, activate replication**.**

To identify compounds that inhibit the formation of NS5A p58, we set up an in vitro assay.
As a source of compounds, we used a collection of proprietary kinase inhibitors with the general structure of ATP-competitive 2,4,5-trisubstituted imidazole inhibitors (fig.8).
NS5A was expressed in Huh7 cells containing the Con1 subgenomic replicon with the adaptive mutation A2199T (Huh7-68). Since it has previously been reported that cellular kinases remain associated with NS5A after immunoprecipitation with specific anti-NS5A antibodies (60), we incubated the cell extract with an NS5A-specific antibody and immunoprecipitated the associated protein complex under native conditions. The immunoprecipitated proteins were then incubated with [γ-33P]ATP in the presence or absence of 5 μM compound. After the reaction, phosphorylated proteins were resolved by SDS-PAGE, and the results are shown in Fig 9.
Comparison of the nonspecific phosphoproteins present in Huh7 cells not expressing the replicon (lane 2) with those obtained from the replicon cells Huh7-68 (lane 3) clearly indicated the production of phosphorylated NS5A.
Most of the phosphates were incorporated into p56, even though a small quantity was visible also in p58 (lanes 3, 4, 6, and 7). Many compounds of the collection had no or only little effect on any of the kinases present in the immunoprecipitation (lanes 4 and 6). A large number of compounds, around 40%, showed 50% or more inhibition.

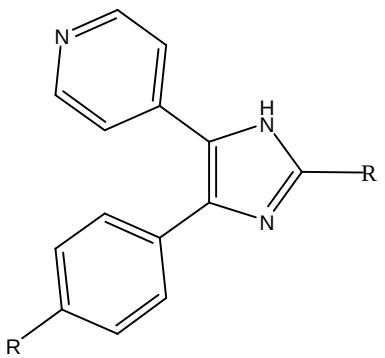


Fig.8
General structure of compounds collection: 2,4,5-trisubstituted imidazole.

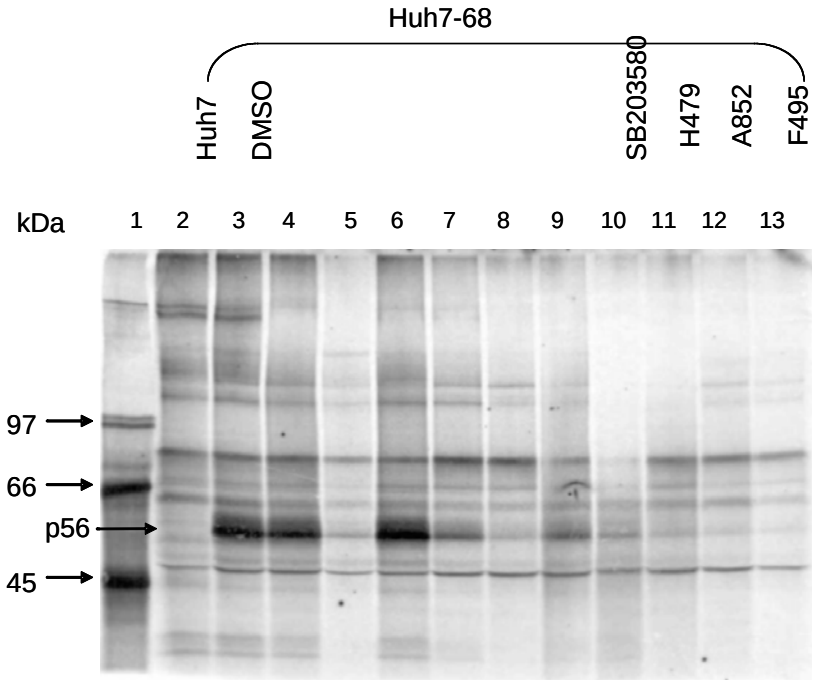


FIG.9

Inhibition of NS5A phosphorylation in vitro. NS5A was immunoprecipitated from Huh7-68 cells and incubated with [γ-33P]ATP as described in Materials and Methods. The phosphorylation of NS5A in the absence (lane 3) or presence of 5 μM concentrations of different compounds (lanes 4 to 13) is shown. As a control, proteins were immunoprecipitated from Huh7 cells (lane 2); the sizes of molecular mass marker proteins are indicated in lane 1.

2.2 Inhibition of NS5A phosphorylation in cell culture by selected compounds.

All selected compounds were evaluated in cell culture to assess their effects on NS5A phosphorylation in the context of live cells and active HCV polyprotein processing by using the vaccinia T7 infection-transfection system. DNA plasmid coding for the wt Con1 HCV replicon was transfected into Huh7 cells, and viral proteins were labeled with [^{35}S]methionine, to monitor protein expression, or with [^{32}P]orthophosphate, to investigate phosphorylation efficiency (Fig.10). Most of the selected compounds had no effect on NS5A phosphorylation in cell culture or affected the expression of NS5A and the other viral proteins without changing the phosphorylation pattern (data not shown). Three compounds selectively inhibited the formation of the p58 form of NS5A (Fig.10, lanes 3 to 5 and 9 to 11). Conversely, no compound was identified which inhibited basal NS5A phosphorylation without affecting NS5A expression. Like many other compounds, the control compound SB203580 inhibited the in vitro phosphorylation of NS5A by more than 50% but had no effect either on protein expression or on protein phosphorylation in cells.
The vaccinia T7 infection-transfection system can be used to study protein phosphorylation during active HCV polyprotein expression and processing. During active HCV replication, however, localization of viral proteins and protein-protein interactions may differ from the vaccinia T7 infection-transfection system, and therefore, the susceptibility of the specific kinase for the inhibitors may change. We incubated Huh7-68 cells with the three indicated compounds and labeled the proteins either with [^{35}S]methionine or with [^{32}P]orthophosphate (data not shown). The results obtained were identical to those already observed with the vaccinia T7 infection-transfection system. Inhibition of NS5A hyperphosphorylation by the three selected kinase inhibitors was further confirmed to not depend on a specific replicon sequence by using cells carrying a replicon that contained an insertion of a lysine after valine 2039 (K@2039), i.e., more than 150 amino acids away from the hyperphosphorylation sites. Also, in this case, the formation of p58 was efficiently inhibited (data not shown).

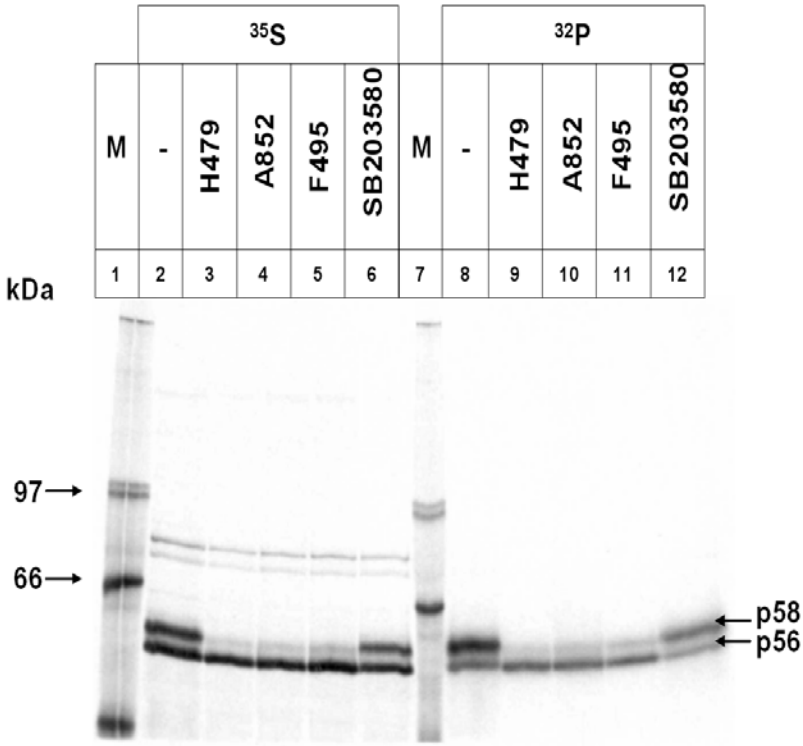


Fig10. Inhibition of NS5A p58 formation in cell culture. NS5A was expressed by using the vaccinia infection-transfection system. The plasmid wt-BLA was transfected for 4 h in 10A-IFN cells, and proteins were labeled either with [35S]methionine (lanes 2 to 6) or with [32P]orthophosphate (lanes 8 to 12) in the presence of DMSO (lanes 2 and 8) or with a 5 μM concentration of compound H479 (lanes 3 and 9), A852 (lanes 4 and 10), F495 (lanes 5 and 11), or SB203580 (lanes 6 and 12). After radiolabeling, the protein extract was prepared, NS5A was immunoprecipitated, and proteins were loaded onto an SDS-7.5% PAGE gel and autoradiographed as described in Materials and Methods.

2.3 Activation of replication of wt Con1 replicon in the presence of kinase inhibitors.

Having identified compounds that specifically inhibit NS5A hyperphosphorylation, we tested their effects on HCV replication in cell culture.
To this aim we used two different methods.
First, we used a subgenomic replicon in which the original neomycin phosphotransferase (*neo*) gene was replaced by the BLA gene (*bla*) (*84*). Cells actively replicating HCV express BLA and show a blue staining after incubation with a diffusible BLA substrate (BLA assay). Replicon RNA was electroporated in 10A-IFN cells, and compounds were added at a concentration of 8 μM 2 h after electroporation. The BLA assay was performed after 4 days of incubation, and results are shown in Fig. 11. As a positive control, we used a replicon containing the adaptive mutation S2204A (Con1 S2204A). This adaptive mutation reduces the formation of NS5A p58, similar to the already published mutation S2204I.
Electroporation of the wt Con1 replicon did not generate any blue cells (Fig.11), whereas the addition of any of the three compounds resulted in the production of blue cells as a consequence of HCV replication (Fig.11, wt-Con 1 + compound). The three selected kinase inhibitors, however, did not induce replication to equal efficiencies. Whereas compound H479 produced more than 50% blue cells, which is comparable to the positive control, the other two compounds generated fewer blue cells. The control compound SB203580 had no effect on HCV replication. To demonstrate that the blue staining is a result of HCV replication and not a result of a longer half life of the electroporated HCV RNA or BLA enzyme, we incubated the cells, in addition to the compounds, with an inhibitor of the HCV RNA-dependent RNA polymerase (RdRP) (+ RdRP-I, bottom) (*85*). In all cases, the numbers of blue cells are significantly reduced. In the case of compound F495, some blue cells remain even in the presence of the RdRP inhibitor. It would be possible that compound F495 stabilizes the HCV RNA and consequently BLA expression to some extent.

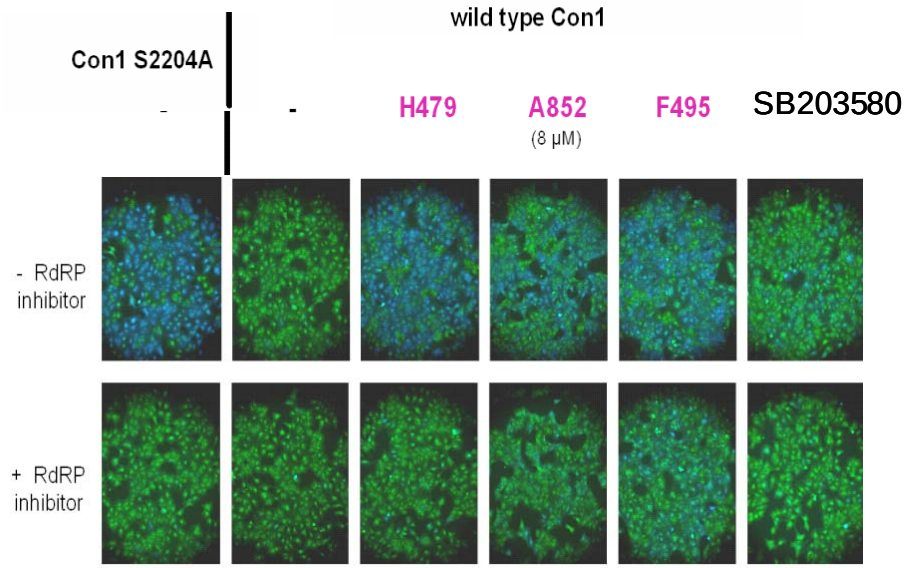


Fig11
Induction of replication of the Con1 wt subgenomic replicon in the presence of kinase inhibitors. RNA was transcribed from plasmid wt type Con1 or Con1S2204A, and 10 µg of RNA was transfected into 10A-IFN cells as described in Materials and Methods. Cells were incubated for 4 days with or without 8 µM compound (top lain) or with or without compound plus 30 µM RdRP inhibitor RdRP-I (bottom lain). The results shown are from the BLA assay, in which blue cell staining indicates active HCV replication.

The previous experiment shows that all three compounds induce the replication of the wt Con1 replicon. We next investigated whether replication is sufficient to allow the detection of viral proteins in the total cell population.
RNA of wt Con1 replicon was electroporated into 10A-IFN cells, and compounds were added 2 h after electroporation. After 4 days of incubation, cells were collected and cellular extracts were assayed for the presence of NS5A by immunoblotting. As expected, no NS5A was visible in untreated cells or in cells incubated with the control inhibitor SB203580.
NS5A could be greatly detected in the presence of compound H479, whereas little protein was visible in cells treated with compound A852 or F495, even though compounds A852 and F495 induced replication (Fig. 12).

The presence of HCV proteins (NS5A in this case) is due to active replication, as the HCV replicon polymerase-minus mutants (wt-GAA) do not show any induced amount of NS5A. One exception is compound F495, which induces a slight increase of even without active HCV replication. The same observation was already made by using the BLA assay (Fig. 11).

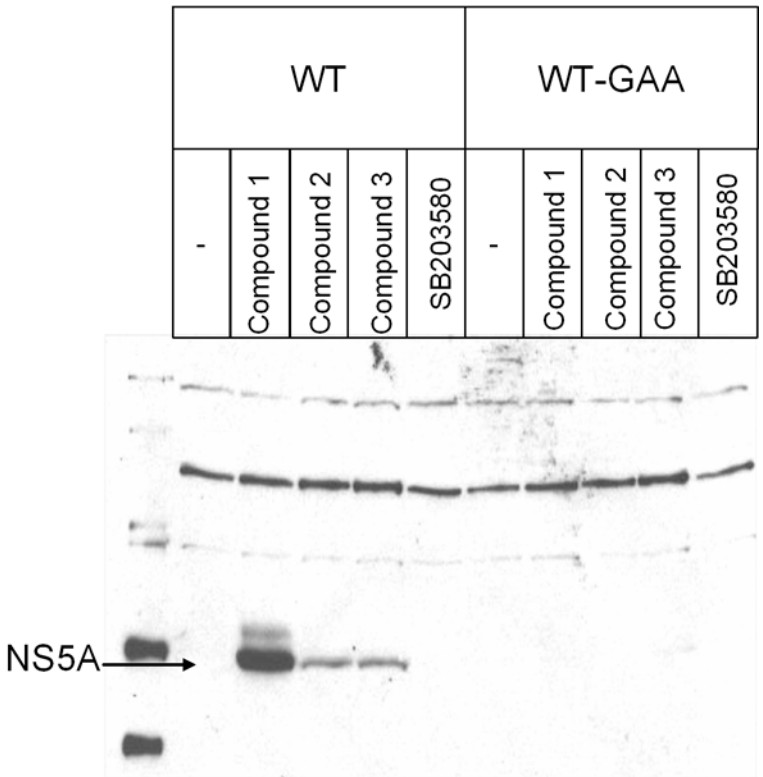


Fig12

Detection of HCV-specific proteins or HCV RNA after induction or inhibition with the specific kinase inhibitors. RNA was transcribed from the plasmids wt, wt-GAA, and electroporated into 10A-IFN cells as described in Materials and Methods. Cells were incubated for 4 days without or with 8 µM concentrations of compound H479, compound A852, compound F495 or SB203580. Western blot analysis of total protein extracts. Cell extract was prepared as described in Materials and Methods, and 50 µg of protein was loaded for each lane. Specific anti-NS5A antibody was used as primary antibodies, and a peroxidase-conjugated antibody (Pierce) was used as a secondary antibody. The Western blot was developed by using the SuperSignal West Pico chemiluminescent substrate (Pierce).

2.4 The Inhibitors of NS5A (p58) hyperphosphorylation inhibit replication of adapted replicons.

Compounds which induce replication of wt replicons have an opposite effect on the replication of already adapted replicons.
To see if the potency of inhibition is different on replicons with several amount of NS5A hyperphosporylated, we electroporated Huh7 cells with, a replicon that contained an insertion of a lysine after valine 2039 (K@2039) (*86*), A2199T and S2004I replicons; the cells are plated and grow in presence of several concentration of H479 or A852 (0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 µM final) and the effect of compounds on viral replication was monitored by Cell-enzyme-linked immunosorbent assay (ELISA).
Both compounds show an EC50 of about 5µM on K@2039 and A2199T replicons, and about 0.6 µM on S2204I replicon (Fig13).
We can conclude that the potency depends on the quantity of hyperphosporylated NS5A: replication of the replicon SI is completely inhibited at a 2 µM concentration of compound A852 and H479; the replicons A2199T and K@2039 that show a higher amount of hyperphosporylation of NS5A were inhibited at 10 µM concentration of compounds.

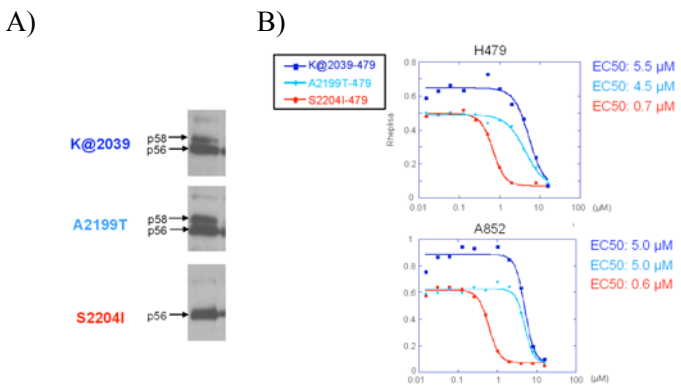


Fig13
The inhibitors of NS5A (p58) hyperphosphorylation inhibit replication of adapted replicons.
A) Adapted replicons S2204I, A2199T, K@2039, bearing different adaptive mutations in NS5A, show in Western blot analysis several amount of NS5A hyperphosporylated.
B) Cells, harboring adapted replicons S2204I, A2199T and K@2039 grow in presence of several concentration of inhibitors, are inhibited at different concentration.

2.5 *Resistant clones*

To demonstrate that the inhibition of replication of adapted replicons by those compounds was correlated to the action on NS5A, we tried to select resistant clones.
Huh7 cells were electroporated with the replicon S2204R (adaptive mutation in the NS5A hyperphosphorylation site), as described in materials and methods, and grown in the presence of 0.8 mg/ml G418 + either 2 μM H479 or 2 μM A852 for several weeks.
Single clones were analyzed by Western Blot and the NS5A region was sequenced. The results are shown in figure 14. Resistance to kinase inhibitor A852 is obtained by 50% of the clones with double-mutation R2204S and A2199T. Other clones resistant to compound A852 and all clones resistant to compound H479 contain a single point mutation in NS5A which converts the adaptive mutation R2204 into G2204.
We can conclude that our compounds directly or indirectly target NS5A, because resistance to inhibitor compounds map to NS5A.
Moreover, in the case of A2199T resistant clones, the resistance to the inhibitors of NS5A hyperphosphorylation is obtained by increased expression of hyperphosphorylated NS5A.
Actually, S2204R adapted replicons convert by double-mutation to A2199T adapted replicons and consequently increase the IC50 values from 0.5 μM (S2204R) to 6 μM (A2199T).
In the case of S2204R adapted replicons that mutate to S2204G adapted replicons we need to carry out further investigations in order to understand the mechanisms of resistance .

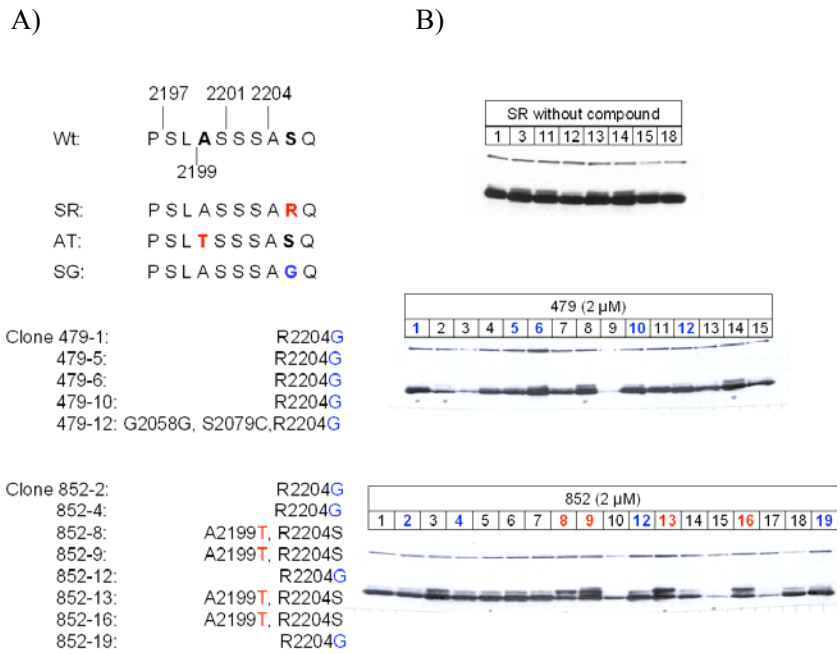


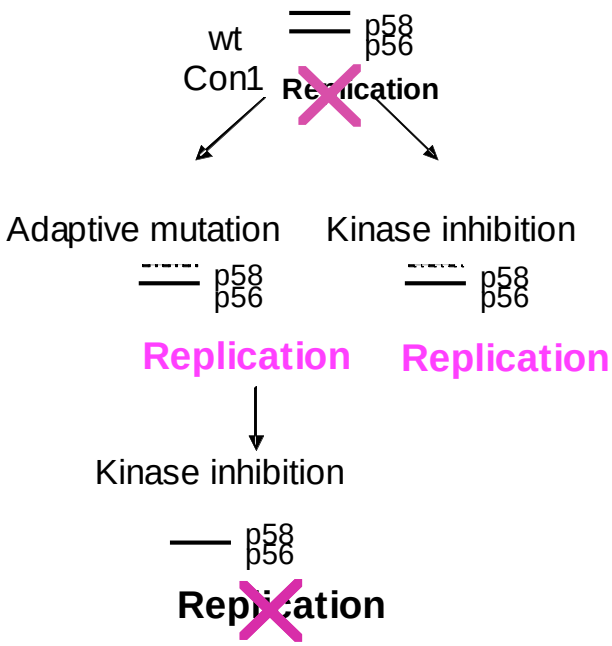
Fig14

Resistance to compounds H479 and A852 maps in NS5A

Huh7 cells were electroporated with the replicon S2204R (adaptive mutation in the NS5A hyperphosphorylation site) and grown in the presence of 0.8 mg/ml G418 + either 2 µM H479 or 2 µM A852 for several weeks. Single clones were analyzed by Western Blot (B) and the NS5A region was sequenced (A). Resistance to kinase inhibitor A852 is obtained by 50% of the clones by increased expression of hyperphosphorylated NS5A p58 (red numbers). Other clones resistant to compound A852 and all clones resistant to compound H479 contain a single point mutation in NS5A which converts the adaptive mutation R2204 into G2204 (blue numbers).

2.6 Outcome of inhibition of NS5A p58 depends on the context

Adaptive mutations which reduce NS5A hyperphosphorylation activate HCV relication and we have demonstrated that reduction of the formation of p58 below a certain threshold by pharmacological inhibition of the NS5A–specific kinase(s) allows replication of non-adapted Con1 HCV RNA in cell culture. On the other hand, complete abrogation of NS5A hyperphosphorylation by the kinase inhibitors abolishes HCV replication (scheme 2).
It seems that a small quantity of p58 is necessary for efficient replication.
Therefore, the cellular kinase which is important for the formation of p58 is an important factor for successful viral replication and might be important antiviral target.



Scheme 2:
We have shown that kinase inhibitors, which reduce NS5A hyperphosphorylation, activate replication**.**
Complete abolishment of NS5A hyperphosphorylation results in inhibition of HCV replication in cell culture.

2.7 NS5A-specific kinase inhibitors H479, A852 and F495 are inhibitors of Casein Kinase I

In order to identify cellular kinases targeted by these compounds we tested their inhibitory activity *in vitro* on a panel of protein kinases **(Table I)**.

	A852 4uM % inhib.	H479 4uM % inhib.	F495 4uM % inhib.		A852 4uM % inhib.	H479 4uM % inhib.	F495 4uM % inhib.
c-RAF (h)	55	36	89	PKCq (h)	0	12	27
MEK1 (h)	6	5	32	Syk (h)	0	60	95
MAPK2 (m)	23	5	77	p70S6K (h)	25	17	40
p38-α (h)	98	100	100	CHK1 (h)	7	42	78
p38-β (h)	90	97	91	AMPK (r)	4	2	81
p38-γ (h)	4	0	0	CDK2/cyclin A (h)	0	0	0
p38-δ (h)	5	0	8	JNK3 (r)	78	57	99
MAPKAP-K2 (h)	9	0	77	cSRC (h)	60	57	98
MSK1 (h)	83	20	60	CK2 (h)	0	7	29
MKK4 (m)	36	27	37	Lck (h)	50	41	92
MKK7b (h)	0	14	46	PRAK (h)	2	0	26
JNK1a1 (h)	12	16	9	PDK1 (h)	2	63	73
JNK2a2 (h)	34	10	65	Lyn (m)	92	59	98
SGK (h)	0	17	10	GSK3b (h)	7	13	0
PKCa (h)	15	20	65	PKA (b)	75	14	76
ROCK-II (r)	70	21	27	PKBa (h)	18	6	27
Fyn (h)	52	49	91	CaMKII (r)	0	0	3
ZAP-70 (h)	1	0	0	CDK1/cyclinB (h)	6	8	12
CHK2 (h)	25	72	100	MAPK1 (h)	20	14	80
PRK2 (h)	13	9	25	CDK2/cyclinE (h)	0	0	23
PKCbII (h)	54	14	78	CDK6/cyclinD3 (h)	0	0	5
PKCg (h)	5	0	20	RSK3 (h)	11	23	42
Blk (m)	41	58	91	IGF-1R (h)	0	0	9
CaMKIV (h)	9	0	0	IR (h)	5	8	2
CDK3/cyclinE (h)	0	8	15	PKBb (h)	50	25	11
CDK5/p35 (h)	0	0	1	FGFR3 (h)	22	10	9
CK1 (y)	91	74	97	PDGFRa (h)	9	6	37
CSK (h)	31	1	3	PDGFRb (h)	5	0	56
IKKa (h)	0	15	0	CDK7/cyclinH/MAT1 (h)	3	18	14
IKKb (h)	0	28	16				

TabI

Inhibitory activity of compounds H479, A852 and F495 on a panel of kinases. Compounds were tested at a fixed concentration of 4 μM and the numbers in the following columns indicate % inhibition of the kinases. Red numbers indicate inhibitory activity of ≥70 %.

Those kinases which are inhibited ≥ 70 % are highlighted in the table. The spectrum of inhibitory activity is different for each compound and only three kinases have been identified which were potently inhibited by all three compounds. Of these, p38-α and p38-β belong to the family of mitogen-activated protein kinases (MAPK) (*87*). The third kinase is Casein Kinase I (CKI) (*88*). Since these compounds were originally designed to specifically inhibit p38-α/β, H479, A852 and F495 were expected to target these two

enzymes. However, SB 203580, a known p38 inhibitor, has no effect on NS5A hyperphosphorylation in cell culture (*89*) and therefore MAPK p38-α/β were excluded from the list of candidate targets.

2.8 Experimental design of Inhibitor Affinity Chromatography

Testing the inhibitors selectivity on a small panel of purified kinases is far from covering the estimated 518 kinases of our genome.
An affinity chromatography approach, using our inhibitors immobilized on a solid matrix, was therefore designed to purify the cellular targets.
The choice of the inhibitor orientation with respect to the matrix is important to ensure a correct interaction of the target protein with the inhibitor. In general a co-crystal structure of the inhibitor with the target protein indicates the area of the inhibitor which can be attached to the matrix. In our case this information is not available and we have to get back to other useful sources. Godl et al. have successfully identified cellular targets of the p38 kinase inhibitor SB203580 (*90*). This inhibitor is structurally related to the NS5A-p58-i which also potently inhibit p38. Consequently, we attached compound H479 and A852 to an epoxy-activated Sepharose matrix at the moiety corresponding to the sulfoxid moiety of SB203580. While compound H479 contained a reactive amine at the desired position, compound A852 had to be modified such that a reactive amine was introduced into this moiety. We tested the activity of this modified compound (A852m) in an NS5A hyperphosphorylation assay und observed a 2-3 fold reduction of activity after introduction of this modification (data not shown). The affinity matrix was prepared as described in Materials and Methods and total cell lysate prepared from 10A-IFN cells was incubated with this matrix at high salt concentration in order to reduce non-specific protein binding. The choice of elution conditions enables us to distinguish between those proteins which bind to the compound with low affinity from those with high affinity. While low affinity binding proteins or ATP-binding proteins (the NS5A-p58-i are competitive with ATP) might be eluted in the presence of ATP alone, high affinity binding proteins require ATP together with the specific compound. We used MAPK p38 as a high-affinity control for the definition of elution conditions. Fig. 15 shows that only the combination of 100 mM ATP together with 1 mM compound is able to efficiently elute the p38 kinase from the affinity matrix.

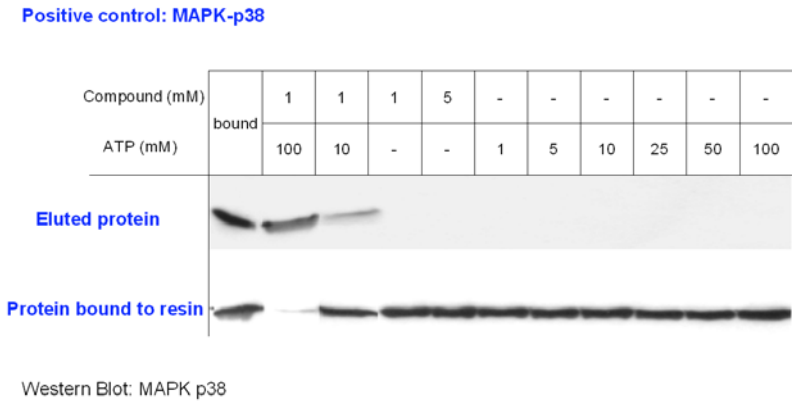


Fig 15
Definition of efficient elution conditions for MAPK p38
Western blot using a p38 specific antibody shows the elution-behavior of p38, used as a positive control.
The most efficient elution of p38 is obtained with 100 mM of ATP and 1 mM compound. Reduction of ATP to 10 mM clearly elutes the protein less efficiently.

2.9 A distinct but overlapping kinase profile is obtained with two NS5A hyperphosphorylation inhibitors

For the identification of cellular targets of the NS5A-p58-i a cellular lysate prepared from $9x10^7$ 10A-IFN cells was loaded onto the inhibitor-affinity-columns. After extensive washing, proteins were first eluted with 100 mM ATP and subsequently with 100 mM ATP plus 1 mM specific compound. Eluted proteins were separated by standard SDS-PAGE and analyzed by mass-spectrometry (Fig. 16 and table 2). The elution profile in the presence of 100 mM ATP with or without 1 mM compound clearly differs indicating that this pre-elution step might have separated low-affinity-binding- or ATP-binding proteins from those proteins with higher affinity for the compounds. Interestingly, most of the bound proteins were kinases belonging to five different kinase families. In addition to this enzyme class, also non-kinase targets such as tubulin, actine, prohibitin, glutamine synthetase and ALDH were found to bind the affinity matrix.
In vitro screening of a selected panel of kinases has shown that only four enzymes, CKI-α, CKI-δ, CKI-ε and MAPK-p38, were efficiently inhibited by three NS5A-p58-i. In this experiment we offered a complex mixture of

cellular kinases and nucleotide-binding proteins present in crude cell extracts. Nevertheless, only CKI-α, CKI-δ, CKI-ε and MAPK p38 bound to both NS5A-p58-inhibitor columns with high affinity and required the presence of compound for efficient elution, thus confirming the previous results. In addition to this common subset of protein kinases, there are also protein targets binding specifically either to compound H479 or to compound A852. For compound H479, RIPK2 was the only kinase present in the specific ATP/compound fraction. Even though we also identified RSK1/2, interaction seemed to be less specific because most of RSK1/2 eluted in the ATP fraction indicating that the amount of protein found in the ATP/compound fraction reflects incomplete elution from the previous elution step. In the ATP fraction of H479, we identified, in addition to the kinase RSK1/2, also JNK1, JNK2 and CaMKIIδ. For compound A852, the high-affinity binding kinases present in the ATP/compound fraction are, in addition to CKI and MAPK-p38, PKA and JNK2. CIT and GAK are present in the low- and high-affinity fractions, indicating a lower affinity of these kinases for the compounds. In addition to CIT and GAK, we also identified GSK3 and JNK1 in the ATP fraction of the A852m-resin.

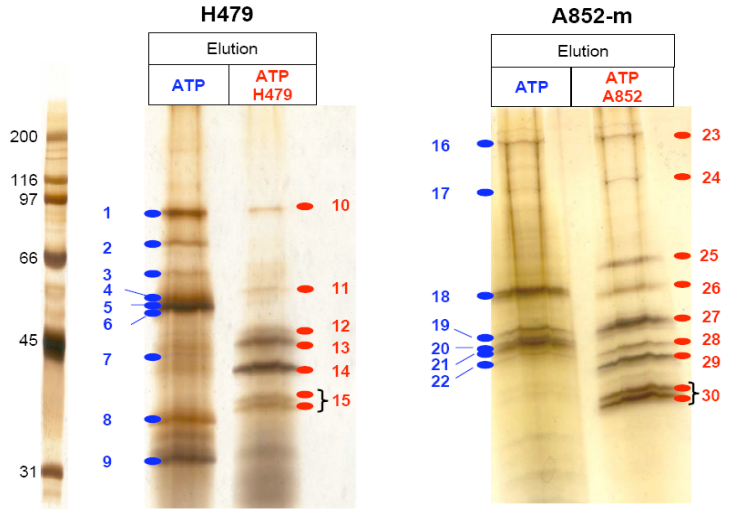


Fig16

Inhibitor Affinity Chromatography with the NS5A hyperphosphorylation inhibitors. Cellular proteins were passed through a column containing covalently bound H479 (left part) or A852m (right part) and proteins were eluted either with ATP or with ATP plus compound. After elution, proteins were concentrated, separated on 10% SDS-PAGE and visualized by silver-staining. Proteins were identified by mass spectroscopy as described Materials and Methods.

Number of protein	Protein name	Swissprot Number
1 / 10	RSK 1 / 2	Q15418/P51812
2	?	
3	?	
4 / 18	Aldehyde Dehydrogenase	P30837
5 / 26	JNK2	P45984
6	CaMK II δ	Q13557
7 / 20	JNK1	P45983
8	?	
9	Prohibitin	P35232
11	RIPK2 (Receptor-interacting serine/threonine-protein kinase 2	O43353
12 / 27	CKI-δ / CKI-ε	P48730 / P49674
13	Glutamine Synthetase	P15104
14 / 29	MAPK p38-α, β	Q16539 / Q15759
15 / 30	CKI-α	P48729
16 / 23	CIT (Citron Rho-interacting kinase)	O14578
17 / 24	GAK (Cyclin G-associated kinase)	O14976
19	GSK3 (Glycogen synthase kinase-3)	P49841
21	Actin	P60709
22 / 28	PKA	P17612
25	?	
26	Tubulin	P68366

Tab 2
Identification of proteins obtained from Inhibitor Affinity Chromatography by MS analysis.

2.10 In Vitro Analysis of the Target Proteins

To confirm the results obtained by mass spectrometry, we repeated the experiment by batch incubation of the affinity resins with the cellular extracts and tested the identity of bound kinases by Western blot analysis. Different affinities of the cellular kinases for the two NS5A hyperphosphorylation inhibitors H479 and A852 were proven by

competition of the compounds in solution during the binding reaction. The results are shown in fig 17. This type of assay measures binding of proteins to the affinity resin, which depends on the intrinsic affinity of the protein for the compound, on the concentration of the compounds linked to the matrix, and on the abundance of the protein in the cellular extract. In addition, we measured the IC50 values of both compounds for some kinases to obtain more quantitative information (Table 3). None of the kinases tested bound to the control resin (17, lanes 2 and 3). CKI-α, CKI-δ, CKI-ϵ, and p38, all kinases binding to both affinity resins with high affinity, behaved similarly and were competed completely in the presence of either H479 or A852 (17, compare lane 5 with lanes 7 and 9 and lane 11 with lanes 13 and 15). This result was expected because the concentrations used for competition (2 mM) were more than 1000-fold the IC50 value of these kinases (see Table 3). Binding and competition of the other kinases reflect the binding affinities already observed in 16 and can be explained with the IC50 value shown in Table 3. PKA binds with high affinity to immobilized A852-m (17, lane 11), whereas no binding is detected to the H479 resin (17, lane 5). Although A852 efficiently competes in binding (2000x IC50, 17, lanes 14 and 15), 2 mM H479 (7x IC50) is not sufficient to complete competition of PKA binding (lanes 12 and 13). In contrast, RSK binds to the H479 resin (Fig. 17, lane 5) and not the A852-m resin (lane 11). Binding affinity of RSK for compound H479, however, is lower than binding affinity of PKA for compound A852, because ATP is sufficient for elution of RSK (16). In addition, 2 mM of either A852 or H479 in solution is sufficient to compete for RSK binding to the H479 resin (17, lanes 6–9). The IC50 values for both compounds are similar, and we assume that the reduction in potency upon introduction of the modification into A852 might explain why RSK does not bind to the A852-m resin. JNK2 represents a third example. JNK2 binds to both affinity resins (fig 17, lanes 5 and 11). However it binds with low affinity to immobilized H479 (elutes with ATP alone) and with higher affinity to immobilized A852-m (ATP/compound fraction) (Fig. 16). The IC50 value for both compounds is in the high micromolar range, and probably high abundance of the kinase within the cell contributes to the binding reaction. Nevertheless, the higher affinity of the kinase for compound A852 is confirmed during the competition reaction, where H479 only partially competes for binding to the A852-m resin (Fig. 17, lanes 12 and 13), whereas A852 is able to compete completely (lanes 14 and 15).

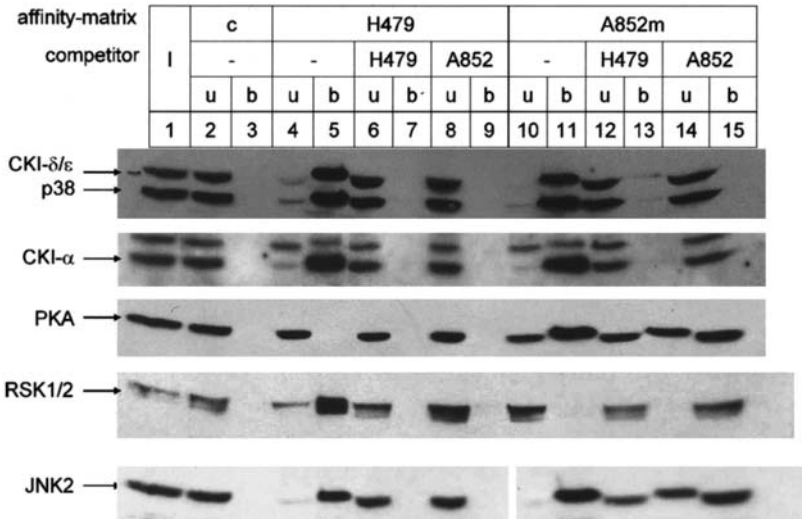


Fig17

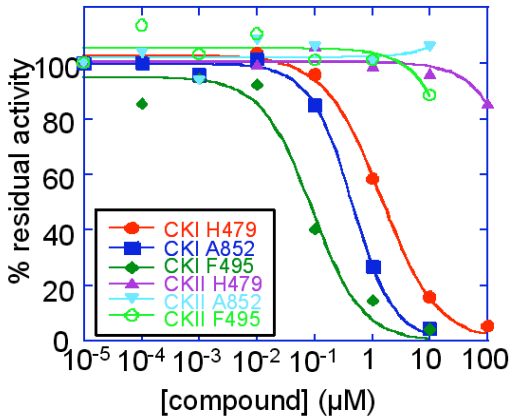
Confirmation of binding and different compound affinities of identified kinases by immunoblot analysis. Cellular proteins were bound in batch either to the control matrix (c) or to the affinity-matrix H479 or A852-m as indicated in the affinity-matrix lane. The presence of competitive compound during the binding reaction is indicated in the competitor lane. Unbound (u) and bound (b) proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with specific antibodies as indicated in the figure. I, input extract.

	H479 (µM)	A852 (µM)
p38 MAPK	**0.028**	**0.024**
CKI-δ	**1.4**	**0.4**
CKI-δ	**1.1**	**1.3**
PKA	**260**	**2**
JNK1	**385**	**175**
JNK2	**404**	**166**
RSK2	**76**	**39**

Tab 3
Calculation of IC50 values (µM) of the NS5A hyperphosphorylation inhibitors H479 and A852 for different cellular kinases in vitro

2.11 NS5A-specific kinase inhibitors H479, A852 and F495 are inhibitors of Casein Kinase I

Since the most promising candidate within the panel of protein kinase and within the cellular target identified by IAC was represented by CKI, we first concentrated our attention on this target.
Yeast CK I was used for the primary screening. To confirm inhibition of the mammalian CKI, we titrated the three compounds on the rat delta-isoform of CKI (CKI-δ). As negative control, we used Casein Kinase II (CKII) which has also been reported to phosphorylate NS5A in vitro. While CKII was not inhibited up to 10 µM by any of the three compounds, all of them efficiently inhibited CKI-δ, with IC50 values of 1.4 µM, 0.4 µM and 0.1 µM for H479, A852 and F495, respectively (Figure 18). Due to high cell toxicity of compound F495, possibly associated with its broad spectrum of action (Table 1), we decided to continue our study only with H479 and A852.



IC_{50} (H479): 1.4 µM

IC_{50} (A852): 0.4 µM

IC_{50} (F495): 0.1 µM

Fig18
H479, A852 and F495 are inhibitors of mammalian CKI-δ *in vitro*. Kinase reactions were performed with recombinant CKI-δ or CKII and synthetic peptides as substrates as described in Materials and Methods. Enzymatic activity was monitored by ^{33}P incorporation using γ-^{33}P-ATP as phosphate donor.

2.12 Inhibitors of CKI reduce NS5A hyperphosphorylation in cell culture

Since CKI was identified as a possible candidate responsible for NS5A phosphorylation, we wanted to investigate the effect of CKI inhibition on NS5A hyperphosphorylation in cell culture.
Several kinase inhibitors are known to be specific for CKI inhibition. The inhibitor CKI-7 is a general CKI inhibitor but penetrates poorly the cellular plasma membrane (*91*), whereas the inhibitor IC261 has been reported to have preferences for the δ - and ε - isoforms of CKI (*92*). We also used SP600125, which was originally identified as a JNK-specific inhibitor but which is, in fact, even more potent on CKI (*93*). A subgenomic Con1 HCV RNA containing the A2199T adaptive mutation (*56*) was electroporated into 10A-IFN cells (*86*) and replication was allowed to proceed for three days. This adaptive mutation was chosen because it does not affect NS5A phosphorylation.
Compounds were then added to the cells at the indicated concentrations, incubated for additional 24 hours and NS5A hyperphosphorylation was detected by Western Blot analysis (Fig. 19). Besides SB203580 we used two other compounds as control. Compound c1 inhibited NS5A phosphorylation *in vitro*, but was inactive in NS5A hyperphosphorylation in cell culture (*89*). Compound c2 is a nonnucleoside inhibitor of the HCV RNA-dependent RNA polymerase NS5B with an inhibitory potency comparable to that of our NS5A-specific compounds (EC_{50} ~5 μM, data not shown). As expected, the NS5A-specific compounds H479 and A852 showed a marked reduction of p58 (lanes 2-3). All CKI inhibitors tested here showed inhibition of NS5A hyperphosphorylation, even though with different efficiency (lanes 5-9). The least active compound was CK1-7, possibly because of its poor cellular uptake. Negative control compounds c1 and c2 (lanes 10-11) did not change the ratio between p56 and p58. This experiment suggests that pharmacological inhibition of CKI causes a reduction of NS5A hyperphosphorylation.

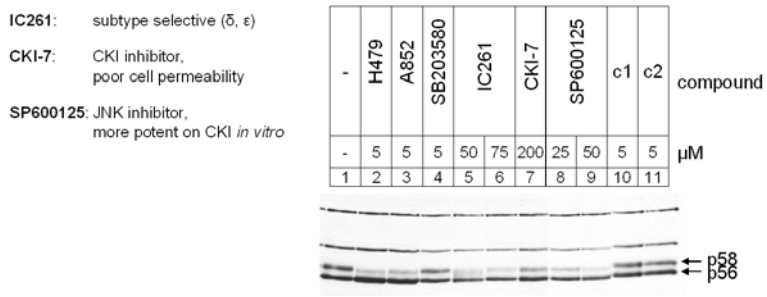
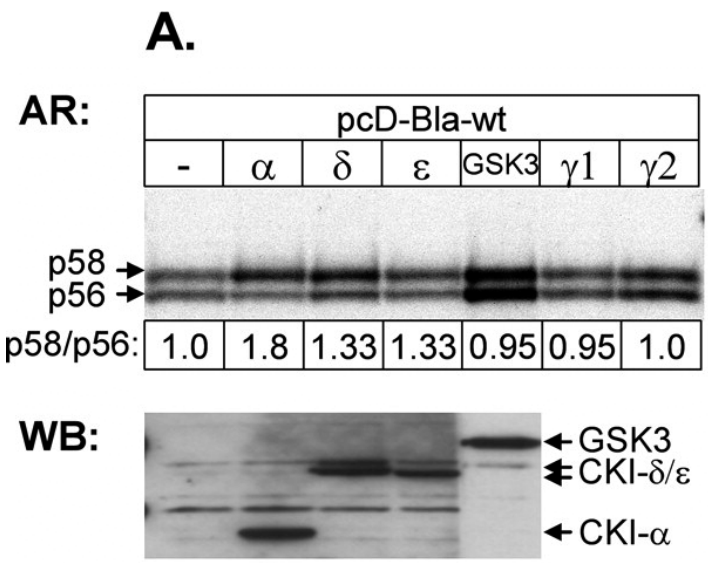


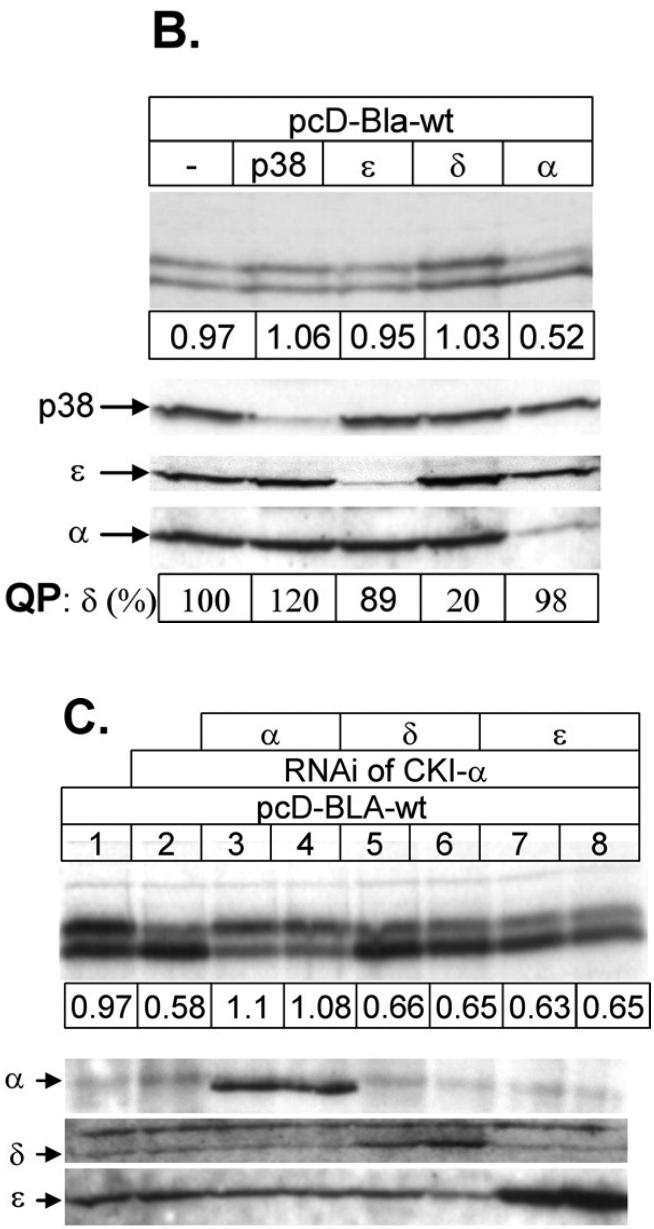
Fig.19
NS5A hyperphosphorylation in cells is inhibited by known CKI inhibitors. In vitro transcribed HCV subgenomic RNA from plasmid Con 1 HCV-A2199T was transfected into 10A-IFN cells. After three days, compounds were added at the indicated concentrations and cells were incubated for additional 24 hours. 50 µg of cell extract was loaded onto a 7.5 % SDS-PAGE and NS5A detected by Western Blot using NS5A-specific polyclonal antibodies. The position of NS5A-p56 and NS5A-p58 is indicated by arrows on the right site of the figure.

2.13 *The CKI-α isoform is important for NS5A hyperphosphorylation*

The results shown above demonstrate that kinases of the CKI family are promising candidates for NS5A hyperphosphorylation. In mammals, the CKI protein kinase family consists of 7 distinct isoforms (α, β, γ1, γ2, γ3, δ and ε) (*94*). In order to test which of the CKI isoforms affects the phosphorylation pattern of NS5A in cells, we performed "gain-of-function" and "loss-of-function" experiments. This way the expression pattern of a single gene is specifically modulated and the effect on NS5A phosphorylation can directly be attributed to this gene product. This is an advantage over the kinase inhibitors and *in vitro* experiments, which can have more or less pronounced off-target activity.
First, we overexpressed the different CKI isoforms in the presence of the HCV nonstructural polyprotein using the Vaccinia T7 infection/transfection system (fig 20A).
As a negative control, we used the non-related kinase GSK3. Proteins were metabolically labeled with 35S-methionine and NS5A was immunoprecipitated with an NS5A-specific antiserum. The only kinases which did not alter the ratio between basally (p56) and hyperphosphorylated NS5A (p58) in this experiment were the CKI γ1- and γ2 isoforms and the

control kinase GSK3. In contrast, overexpression of the α- and δ- isoforms clearly increased the amount of NS5A-p58. A slight increase could also be observed for CKI-ε (see p56/p58 ratio). Overexpression of the α-, δ- and ε-isoforms and GSK3 was confirmed by Western Blot, lower panel), whereas overexpression of the γ-isoforms was confirmed by quantitative PCR due to the lack of appropriate antibodies (data not shown).
Next, we tested whether the opposite effect could be observed upon silencing of individual kinase genes (fig 20 B). Since we could not detect any change in NS5A phosphorylation upon overexpression of the CKI-γ isoforms, we focused our attention on the α, δ, and εisoforms. 10A-IFN cells were transfected with siRNAs directed to CKI-α, δ, ε or p38-α as negative control, After 48 hours, the HCV subgenomic replicon was expressed using the Vaccinia T7 infection/transfection system and NS5A was metabolically labeled and immunprecipitated .A clear reduction of NS5A hyperphosphorylation was observed only upon silencing of CKI-α expression, suggesting that CKI-α is the isoform responsible for the modulation of NS5A hyperphosphorylation in cells. The silencing efficacy for each kinase was monitored by Western Blot analysis or by RT PCR.





Fig

CKI-α plays an important role in NS5A hyperphosphorylation. (A) Overexpression of CKI-α, CKI-δ, and CKI-ε increases NS5A-p58 levels. Plasmid pcD-Bla-wt (2 μg) was transfected together with plasmids expressing the indicated kinases (each 1 μg) in 10A-IFN cells, and proteins were expressed using the vaccinia virus-T7 infection/transfection system. The proteins were labeled, and NS5A was immunoprecipitated from 20 μg of total protein

extract. The proteins were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and an autoradiogram (AR) is shown in the upper panel. The lower panel shows a Western blot (WB) of the specific kinases. CKI-α was detected with α-FLAG antibody. NS5A and the kinases are indicated. (B) RNAi of CKI-α decreases NS5A hyperphosphorylation. The indicated kinases were silenced in 10A-IFN cells as described in Materials and Methods. Forty-eight hours after siRNA transfection, 2 μg of pcD-Bla-wt was transfected, and the proteins were expressed, using the vaccinia virus-T7 infection/transfection system. The proteins were labeled, and NS5A was immunoprecipitated. The upper panel shows the autoradiogram. Silencing of the different kinases is shown in the Western blot for CKI-α/ε and p38 and by quantitative RT-PCR for CKI-δ in the lower panels (QP). The numbers indicate mRNA expression levels of CKI-δ with respect to that of untransfected cells (100%). (C) Overexpression of CKI-α rescues inhibition of NS5A hyperphosphorylation. CKI-α expression was silenced as described for panel B. After 48 h, 2 μg of pcD-Bla-wt and 0.5 μg (lanes 3, 5, and 7) or 1 μg (lanes 4, 6, and 8) of plasmids expressing the indicated kinases were transfected. Proteins were expressed and labeled as described above, and NS5A was immunoprecipitated. The upper panel shows an autoradiogram. The lower panels show a Western blot of the overexpressed kinases. CKI-α was detected with α-FLAG antibody.

In order to further strengthen this result, we investigated whether NS5A hyperphosphorylation could be rescued by overexpression of the different CKI isoforms in a CKI-α-silenced cellular background (fig 20 C).
The CKI-α isoform was silenced as shown and the HCV replicon was expressed together with the different CKI isoforms using the Vaccinia T7 infection/transfection system as described above. As expected, silencing of CKI-α reduced NS5A-p58 (compare lanes 1 and 2). Upon concomitant overexpression of CKI-α, a clear increase of NS5A hyperphosphorylation was observed (lanes 3-4), whereas overexpression of CKI-δ or CKI-ε did not significantly affect the NS5A p58:p56 ratio (lanes 5-8). Overexpression of the respective kinases is shown in the bottom panels.

2.14 *Overexpression or silencing of CKI-α affects the potency of the NS5A-specific kinase inhibitors*

Another set of experiments was performed in order to further demonstrate that the CKI-α isoform is the target of the NS5A kinase inhibitors. We measured the effective compound concentration required to inhibit 50 % of NS5A hyperphosphorylation in cell culture (EC_{50}). We anticipated that overexpression of the target kinase should increase the EC_{50}, whereas silencing of this kinase should decrease it. In all cases HCV proteins were expressed using the Vaccinia infection/transfection system and compound

H479 was present in increasing concentration during HCV protein expression.
The EC_{50} for compound H479 was between 1 and 2 μM (Fig. 21**,** panel A). Upon overexpression of CKI-α, the EC_{50} increased and could be estimated around 4 μM (Fig. 21, panel B). An opposite effect could be observed upon silencing of the kinases (Fig. 21**,** panel C). The EC_{50} clearly dropped below 1 μM in a CKI-α silenced cellular background. Overexpression and silencing of CKI-δ or CKI-ε isoforms did not show the same correlation between kinase expression level and the EC_{50} of the compound (data not shown).

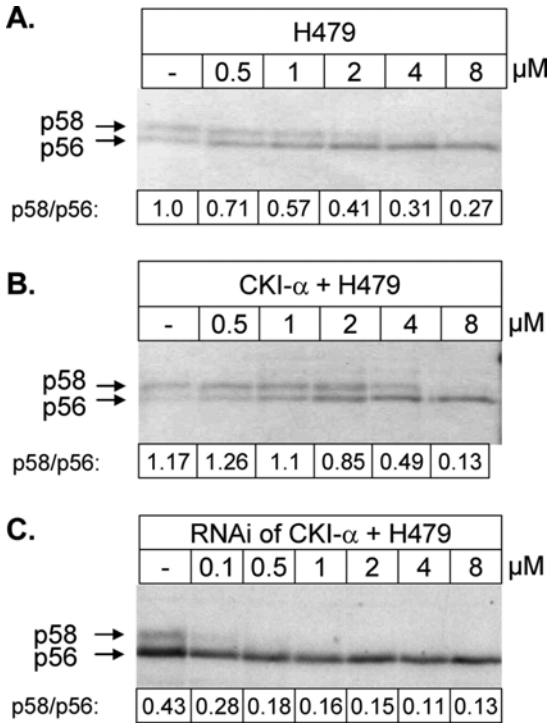


Fig.21
The EC50 of compound H479 depends on the expression level of CKI-α. pcD-Bla-wt (2 μg) was expressed using the Vaccinia infection/transfection system either alone (panel A) or together with 1 μg plasmid expressing the indicated kinases (panel B). In panel C, the indicated kinases were silenced as described in Materials and Methods and pcD-Bla-wt (2 μg) was expressed 48 hours after RNAi. H479 was added at the indicated concentrations, proteins were labeled and NS5A was immunoprecipitated as described in Materials and Methods . The position of NS5A p56 and p58 is indicated.

2.15 Inhibition of HCV replication upon silencing of CKI

We have shown that the NS5A-specific compounds inhibit HCV replication. In this work we have demonstrated that CKI-α is targeted by compound H479 (Fig. 21). We next investigated whether HCV replication is inhibited as a consequence of reduced expression of CKI-α. In order to perform this experiment we used HuH7 cells which stably express an HCV subgenomic replicon containing the adaptive mutation S2204R. This mutation behaves similarly to the S2204A mutation, which shows a reduced formation of hyperphosphorylated NS5A (*89*). We chose this adaptive mutation for the following experiments because it is the replicon, like S2204I replicon, more potently inhibited by the compound H479 (Fig.13). Mock-transfected cells or siRNA-transfected cells were collected 1, 3, and 5 days after electroporation and controlled for HCV RNA and silencing efficiency of the kinase by quantitative PCR (Fig 22). mRNA levels of HCV or CKI-α in the mock-transfected cells were arbitrarily set to 100%. Throughout the duration of the experiment, the mRNA levels of CKI-α in those cells transfected with the specific CKI-α siRNA remained below 30% of that of the mock-transfected cells (Fig 22, right panel). At the same time points, HCV RNA slowly decreased and reached a minimum of 40% with respect to that of the mock-transfected cells at day 5, which means a 60% inhibition. This experiment shows that reduction of CKI-α expression results in inhibition of HCV replication.

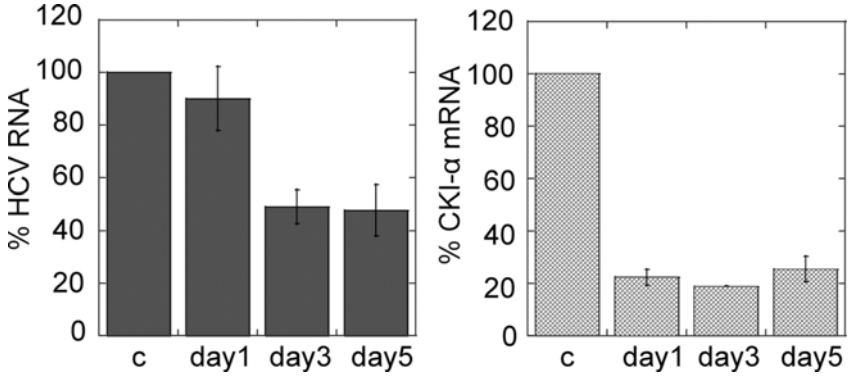


Fig22
Silencing of CKI-α HCV replication. CKI-α was silenced by transfection of siRNA in SR3 cells. After 1, 3, and 5 days, RNA was isolated and mRNA for CKI-α (right panel) and HCV RNA (left panel) were detected using quantitative PCR. Shown are the relative RNA quantities expressed as percentages of those of the mock-transfected control cultures (c) at each time point. The data shown represent the averages of the results of three independent experiments, and the error bars indicate the experimental standard deviations.

2.16 RNA Interference Experiments Confirm CKI-α as the Unique Target Kinase Important for NS5A Hyperphosphorylation

The inhibitor affinity chromatography has revealed additional cellular kinases that could be potential targets for the NS5A hyperphosphorylation inhibitors. The most effective way to test whether these kinases influence NS5A hyperphosphorylation turned out to be RNA interference of the single kinases. Kinases were silenced for 48 h, and the HCV polyprotein was expressed in this cellular background using the vaccinia T7 infection/transfection system. Phosphorylation of NS5A after silencing of the indicated kinases is shown in Fig. 23. The typical 1:1 ratio of NS5A-p56 and p58 remains constant in all cases except after silencing of CKI-α, where a clear decrease of p58 can be observed. Silencing efficiency varied between 9% in the case of PKA mRNA and CKI-ε mRNA and 35% for CaMK mRNA as calculated by quantitative PCR. We cannot exclude the possibility that residual activities of the kinases after silencing might still be sufficient for complete NS5A hyperphosphorylation; however, under this experimental setting the only kinase that changes phosphorylation pattern of NS5A is CKI-α.

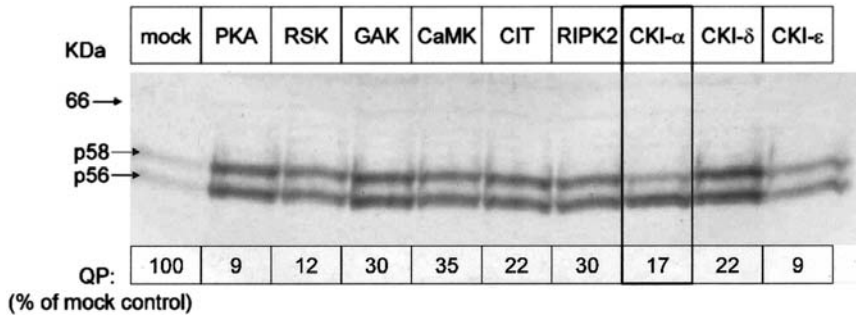


FIG23. CKI-α is the only target kinase important for NS5A hyperphosphorylation. The indicated kinases were silenced in 10A-IFN cells as described under "Experimental Procedures." 48 h after siRNA transfection, 2 μg of pcD-Bla-wt were transfected, and proteins were expressed using the vaccinia T7 infection/transfection system. Proteins were labeled, and NS5A was immunoprecipitated as described. Shown is an autoradiogram. Efficiency of silencing of the different kinases is shown at bottom by quantitative reverse transcription-PCR (QP). Numbers indicate mRNA expression level of the different kinases with respect to mock-transfected cells (100%).

2.17 Apparent hyperphosphorylation of NS5A occurs upon incubation with CKI-α in vitro

The data shown above demonstrate that CKI-α is a target of both NS5A hyperphosphorylation inhibitors and that it is required for NS5A hyperphosphorylation. These data, however, do not indicate whether NS5A is a direct substrate of CKI-α or whether CKI-α is an upstream kinase of a pathway finally resulting in NS5A hyperphosphorylation. We performed an in vitro kinase assay, in which we incubated several kinases with NS5A purified from E. coli as a substrate (Fig. 24). Besides CKI-α and CKI-δ, we used CKII and PKA as control kinases, both kinases which have been reported to phosphorylate NS5A in vitro (61), (62). The most interesting result of this experiment is the appearance of a slower migrating band (p58*) upon incubation with CKI. P58* does not appear with CKII or PKA (doubling the enzyme concentration of CKII does not result in the production of a second band, data not shown). The reaction is slowed down in the presence of compound H479. It can be observed that the phosphorylation state of NS5A after 4 hours of incubation in the presence of compound is comparable with a 20 minute incubation without compound. We have introduced the S2204R mutation in NS5A, and the effect on NS5A hyperphosphorylation is clearly visible when expressed from the HCV polyprotein in cells (see fig 24 C, lane 4). Interestingly, the same effect could also be observed in the in vitro kinase reaction, in which only NS5A is present (Fig 24 C, lane 2). This observation supports the idea that the in vitro reaction might reproduce NS5A phosphorylation/hyperphosphorylation observed so far only in cells. The difference in migration of NS5A expressed in E. coli is probably because of the N-terminal deletion of the amphipathic helix, which was removed to facilitate expression and purification (Fig 24 A). As this deletion is the only significant difference between NS5A expressed in cells and NS5A expressed in E. coli, we tested whether this deletion enables NS5A hyperphosphorylation in cells even when expressed as a single protein and not in the context of the HCV polyprotein. However, as demonstrated in Fig 24 C, lanes 5–7, hyperphosphorylation of NS5A in cells requires the presence of the HCV nonstructural proteins, independent of the N-terminal amphipathic helix.

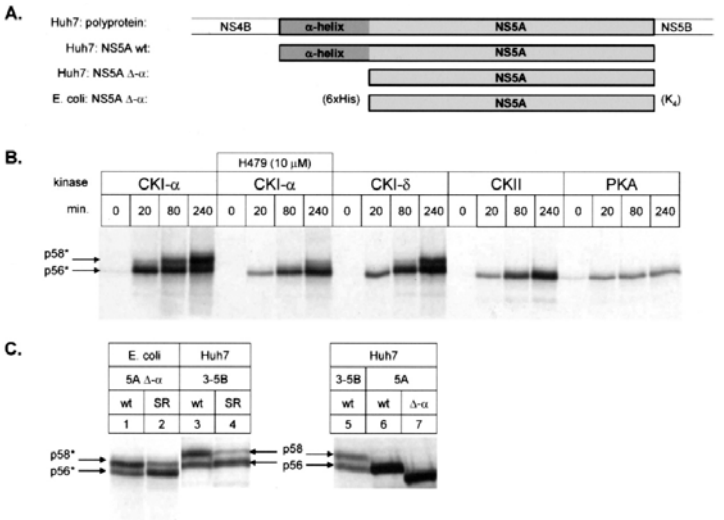
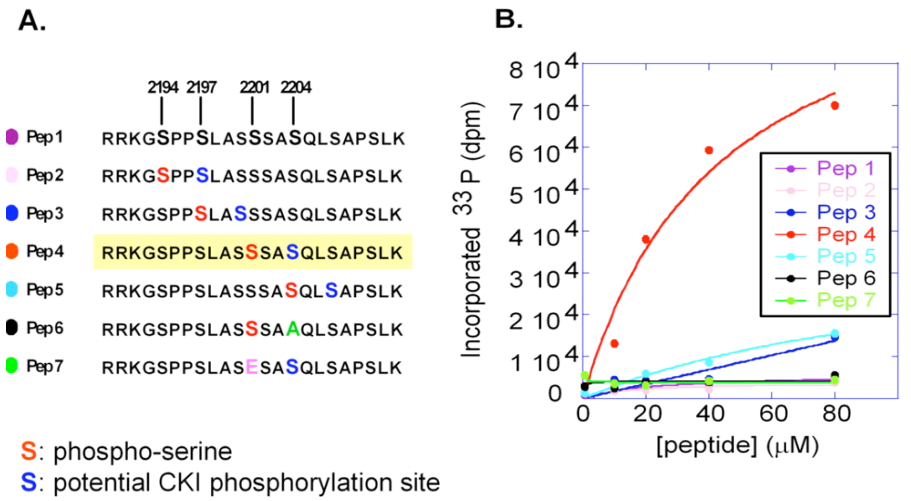


Fig.24

NS5A is hyperphosphorylated in vitro by Casein Kinase 1.

A, schematic presentation of the NS5A sequence used for expression in Huh7 cells or in E. coli. Δ-α, N-terminal deletion of amphipathic helix. Purified proteins may contain a histidine tag at the N terminus (6xHis) or a lysine tail at the C terminus (K4) as described under "Experimental Procedures." B, production of NS5A-p58* in vitro by CKI. Purified NS5A expressed in E. coli was incubated with CKI-α, CKI-δ, CKII, or PKA in the presence or absence of 10 μM H479 for 0, 20, 80, or 240 min at room temperature with [γ-33P]ATP as described under "Experimental Procedures." Proteins were separated by SDS-PAGE and shown is the autoradiogram. The position of in vitro labeled NS5A is indicated as p56* and p58* on the left. C, in vitro NS5A phosphorylation mimics phosphorylation in cells however, it does not require the presence of HCV nonstructural proteins. Purified NS5A was phosphorylated either in vitro by CKI-α (lanes 1 and 2) or was expressed in Huh7 cells either alone (5A, lanes 6 and 7) or in the context of the HCV polyprotein (3–5B, lanes 3–5) using the vaccinia T7 infection/transfection system. NS5A was expressed either from the Con1 wild type sequence (wt, lanes 3, 5, and 6), from an S2204R-mutated sequence (SR, lane 4), from a sequence containing an N-terminal deletion of the amphipathic helix (Δ-α, lanes 1 and 7) or containing both the N-terminal deletion and the S2204R mutation (lane 2). Proteins were labeled, and NS5A was immunoprecipitated as described.

CKI prefers substrates which have previously been phosphorylated within the consensus sequence S/T(P)-X_{1-2}-S/T (*95*). This condition is fulfilled within the NS5A amino acid sequence around the hyperphosphorylation sites, which contains many potential phospho-serine residues. We designed five peptides spanning the region from residue G2193 to K2212, either unphosphorylated or phosphorylated at a single defined serine residue. The serine residues that were phosphorylated are either one of the identified hyperphosphorylation sites S2197, S2201 or S2204, or the serine residue responsible for basal phosphorylation of NS5A, S2194 (*67*) (Fig. 25A). Interestingly, all phosphorylated serine residues within this peptide sequence are situated within a possible CKI consensus sequence. These peptides were offered as substrates to CKI in an *in vitro* kinase assay and the results are shown in Fig. 25B. The best substrate peptide is peptide 4. Efficient phosphorylation of peptide 4 depends on the presence of phospho-S2201 (compare peptide 1 with 4). Comparison of the sequence of peptide 4 with the CKI consensus sequence suggests that serine S2204 is phosphorylated by CKI. This assumption was confirmed using peptide 6. In this peptide S2204 was substituted by alanine, resulting in complete abolition of CKI activity (Fig. 25B). Similar experiments with recombinant CKII showed no activity on these peptides (data not shown). It can be concluded that S2204 preceded by the pre-phosphorylated S2201 residue serves as an efficient CKI substrate.



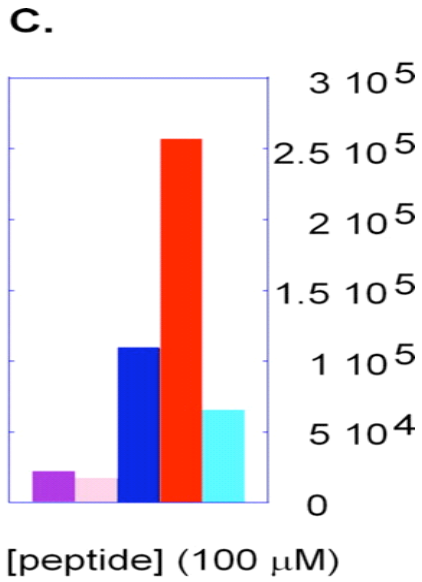


Fig 25. Efficient phosphorylation of a NS5A-peptide requires pre-phosphorylation of S2201. Seven different peptides spanning the region of the NS5A hyperphosphorylation sites were incubated with CKI as described under "Experimental Procedures." A, schematic presentation of the seven peptides. Phosphorylated serine residues are shown in red. Putative substrate serine residues of CKI within the consensus sequence are shown in blue. The S2204A mutation is shown in green, and the S2201E mutation is shown in pink. B, in vitro activity of CKI-δ on NS5A peptides at increasing peptide concentrations. C, in vitro phosphorylation of peptides 1–5 by CKI-α at a single peptide concentration. 33P incorporated in the peptides is shown as disintegrations/min on the y axis at various concentrations of the substrate peptides (x axis).

3. Discussion

The aim of this work was the identification of the cellular kinase(s) which are required for the hyperphosphorylation of NS5A. Identification and characterization of the cellular kinases would contribute significantly to further understanding the HCV life cycle and could possibly lead to the identification of novel therapeutic strategies for the treatment of hepatitis C patients.

Since the identification of adaptive mutations in the HCV subgenomic replicon, it has been noted by several groups that the regulation of the phosphorylation state of NS5A play a very important role in HCV replication. In fact many and among the most effective adaptative mutation happen on the serine residues, which are involved in NS5A hyperphosporylation. This observation suggested that a large amount of the hyperphosphorylated form of NS5A, p58, might not be necessary for replication in cell culture. We went one step further and we demonstrated that high quantity of p58 is not only not necessary but also deleterious for the replication of the Con1 replicon. To demonstrate this, we reduced the formation of NS5A p58 by chemical means, without altering the wt sequence of the Con1 HCV genome. An in vitro assay was set up which enabled us to screen a collection of small molecules for their inhibitory activity on kinase(s) responsible for NS5A phosphorylation. The principle of this in vitro assay is based on the fact that the specific kinase(s) remain stably associated with NS5A after immunoprecipitation *(60).*

In replicons containing the A2199T adaptive mutation, p56 and p58 are present in equal amounts. Nevertheless, in the in vitro assay, radiolabeled phosphate is incorporated predominantly in p56. At the beginning of this work we thought that the explanations for this fact could be the hyperphosphorylation on NS5a is a highly regulated process which requires the expression of an intact NS3-5a polyprotein and a correct polyprotein processing (*70*, *71*). At the end of this work we demonstrated that the hyperphosphorylation of NS5a can occur also in vitro, so probably the timing (45') of this reaction was not enough to see 1:1 ratio between P56:P58.

The low stringency of the *in vitro* assay produced a hit rate of about 40%, with a threshold of at least 50% inhibition of NS5A phosphorylation. As a consequence, a large number of compounds showed the same in vitro activity as the three selected compounds but were inactive in cell culture. There are several explanations for the difference between in vitro activity

and activity in cell culture. One possibility could be limited penetration of the compounds into the cells. Alternatively, a high potential of the compounds for protein binding or instability in the culture medium or within the cell could limit the access to the compound. All compounds tested were designed to be active-site inhibitors and competitors of ATP. Another possible explanation would be that the affinity of the compound for the kinase is too low to compete with the relatively high concentrations of ATP within the cell. Retesting the selected compounds in cell culture with the vaccinia T7 infection-transfection system identified three active compounds. The formation of p58 is inhibited, whereas the basal phosphorylation of p56 remained unchanged (Fig10).

The identification of three compounds that selectively inhibited NS5A hyperphosphorylation, but not basal phosphorylation, allowed us to verify whether a reduction of the amount of NS5A p58 in the context of the Con1 wt sequence could be sufficient for the activation of subgenomic replication in cell culture. It turned out, in fact, that all three active kinase inhibitors were also active replication inducers (Fig. 11-12). Addiction of any of the three compounds resulted in an activation of HCV replication (blue cells un bla assay and deletion of HCV protein in western blot). However the efficiency of the three compounds is different even if the rate of inibition of P58 formation is very similar.

The different degrees of replication efficiency induced by compound H479 and compounds A852 and F495 are probably not a consequence of different potency in the inhibition of p58 formation, which seems to be comparable at 5 μM (Fig. 10). This apparent difference could be explained by considering the cytotoxicity displayed by compound A852 and especially by compound F495 at concentrations of 8 μM or more (data not shown). During the experiments designed for the detection of inhibition of p58 formation, the cells are exposed to 5μM compound for several hours, whereas they are exposed for 4 days at 8 μM during the induction of replication. It is therefore possible that inhibition of NS5A hyperphosphorylation is necessary for the induction of replication, but different side effects of the compounds may induce or block other pathways which may have antagonistic effects on the induction of replication. For this reason the spectrum of cellular kinases inhibited by these agents and whether any of these kinases is directly or indirectly responsible for NS5A hyperphosphorylation needs to be clarified.

There is an additional interesting observation we would like to discuss. Figure 10 clearly shows that treatment of cells with compound H479, A852, or F495 results in a substantial reduction of p58 but is not completely

abolished. Two lines of evidence indicate that complete prevention of p58 formation eliminates replication. (i) Blight et al. (*56*) demonstrated that the mutation S2204I is a very efficient adaptive mutation, whereas the combination of this mutation together with a second adaptive mutation within the hyperphosphorylation sites of NS5A (S2197P) completely abolished replication. (ii) Incubation of cells containing a replicon with the adaptive mutation S2204A with one of our kinase inhibitors also abolishes HCV replication (Data not shown). In both cases, mutation at one hyperphosphorylation site significantly reduces the quantity of p58 and the second event, either a second mutation or inhibition by our compounds, further reduces p58 formation and results in a complete block of replication. This observation suggests, contrary to what has been thought to date, that low amounts of p58 may in fact be necessary for replication and that compounds that completely inhibit NS5A hyperphosphorylation could have antiviral activity in vivo.

To further investigate the inhibition activity of our compound, we studied the efficiency of replication of S2004I, K@2039 and A2199T replicons at several concentrations of compounds (fig.13). We chose those replicons because they show different amount of NS5A hyperphosphorylated. In the case of A2199T and K@2039, there is more NS5A hyperphosporylated than in S2204I. (fig.13A). The replicon bearing different adaptive mutations on NS5A were inhibited at different concentrations of the compounds. The replicon with less hyperphosphorylated NS5A, S2204I, was completely inhibited at 2μM of both compounds. The two replicons with more NS5A hyperphosphorylated, A2199T and K@2039, were completely inhibited at 10μM of both compounds. (fig.13B). Therefore, replication of HCV subgenomic replicons bearing different adaptive mutations in NS5A and consequently different p58 expression levels can both be inhibited by p58-specific kinase inhibitors. Our findings support a role for NS5A p58 in down-regulating HCV RNA replication in cell culture, but, based on the present data, we cannot exclude that the compound(s) we identify act via the inhibition of the phosphorylation of cellular protein(s) regulating HCV RNA replication and that inhibition of p58 is not the actual cause of regulation replication.

To exclude this eventuality, we selected clones resistant to p58-specific kinase inhibitors, as described in materials and methods. We chose S2204R because, it is therefore more sensible to compound treatment and concentration well below cytotoxicity may be used. First we analyzed NS5A of single clones by Western blot and noticed that there was an increased

expression of hyperphosphorylated NS5A in some of the resistant clones to A852. Then we sequenced NS5A region of all clones and the 50% had a double mutation R2204S and A2199T. This result confirm our hypothesis, because an adapted clone with a few hyperphosphorylated NS5A reverted to another adapted clone with more NS5A hyperphosphorylated. The other resistant clones to compound A852 and all clones resistant to compound H479 contained a single point mutation in NS5A which converted the adaptative mutation R2204 into G2204. We need to further investigate the significance of this mutation. Moreover, all NS5A sequences investigated so far contained mutations, indicating that NS5A is likely to be a target of the compounds and that this activity is correlated with HCV replication.

The conclusion of this part of our work is that little changes in the amount of hyperphosphorylated NS5A in cell culture can be very important for the HCV replication and we think that there are differences between *in vivo* and *in vitro* systems in the regulation of 58 formation.
Production of high levels of p58 may be an artifact of the subgenomic construct. The expression of the nonstructural proteins is driven by the strong encephalomyocarditis virus internal ribosome entry site, and the amount of NS5A p58 during infection in vivo could be significantly lower. Alternatively, the specific kinases and, thus, the formation of p58 are expressed or regulated differently in cell lines with respect to the liver, thus disturbing the correct equilibrium between p56 and p58. Different expression of cellular factors could also explain the strict host dependence of HCV for replication in cell culture. For these reasons, we though it would be of great interest to identify the kinases which are responsible for the formation of p58. A cellular system in which the expression of this kinase can be regulated would be an ideal system for the study of the replication of HCV containing the original, infective wt sequence.

To identifiy the cellualar kinases involved in NS5a hyperphosphorylation we used the three previously identified NS5A hyperphosphorylation inhibitors as a tool to screen a limited panel of cellular kinases in vitro. The most promising hit within the panel was represented by the yeast protein kinase CKI, which was potently inhibited by all three compounds. The obvious weakness of testing NS5A specific inhibitors on a panel of 60 kinases is the limited number of potential targets, considering the existence of more than 500 kinases within a cell. So we adopted also an alternative methodology, the Inhibitor Affinity Chromatography (IAC), to find other targets not

present in the previous screen may be identified and evaluated for their effect on NS5A phosphorylation.
Most of the proteins binding to the affinity matrix are kinases, even though non-kinase targets could be identified. These non-kinase targets like tubulin, actine, prohibitin and ALDH bind nucleotides such as ATP or NADH and might associate with the affinity matrix due to their high abundance within the cell. In fact most of these proteins eluted in the presence of ATP alone indicating that the binding is due to their affinity for nucleotides rather than for the specific compounds.
MAPK p38 and CKI, those kinases which we have previously been shown to be inhibited in vitro by all three NS5A-p58-i, also bind to both inhibitor-matrixes with high affinity, thus confirming that their affinity is high enough to compete with all other cellular kinases and nucleotide-binding proteins. Affinities of binding varied between the low nanomolar range up to the high micromolar range. The fact that protein kinases with IC50 as high as 400 μM were able to stably associate with the affinity resin suggests that their isolation may result from higher cellular abundance. High local concentration of compound on the resin might also facilitate binding. In addition, while calculation of the IC50 is performed in the presence of 125 μM ATP, intracellular ATP is diluted out during extract preparation thus the binding reaction is performed in the absence of competing ATP.
As a fact some of the kinases identified by us have already been isolated using the inhibitors SB203580 or pyrido[2,3-d]pyrimidine (*90*),. The authors concluded that these two inhibitor classes have an overlapping set of cellular protein kinase targets. The NS5A hyperphosphorylation inhibitors are structurally related to SB203580 (see general structure in Fig. 8) and it is therefore not surprising that this set of kinases associates with the NS5A kinase inhibitors. All kinases which were identified in the high-affinity fractions of the compounds were tested for their effect on NS5A hyperphosphorylation using the method of RNAi. With the exception of CKI-α, silencing of none of the other kinases changed the NS5A phosphorylation pattern (Fig 23). One can however not exclude the possibility that residual kinase activity of the other kinases after silencing is still sufficient for NS5A phosphorylation. Nevertheless, the fact that p38 and probably also RIPK2, CIT and GAK are also efficiently inhibited by these inhibitors might account for undesired off-target activities. PKA is the only kinase tested in this work which showed a clear difference in potency between the two compounds A852 and H479. At 8 μM concentration, a concentration sufficient to inhibit NS5A hyperphosphorylation and activate

replication of wild type Con1 RNA in cells, PKA is significantly inhibited by A852 (IC50 1 μM), but not at all by H479 (IC50 270 μM). It was shown previously that A852 activates replication of wt Con1 HCV RNA less efficiently than H479 (*89*) and this difference might be explained by selective inhibition of PKA by A852. As a general observation one can state that A852 inhibits kinases with lower IC50 values compared to H479 and competes more efficiently in solution (Tab 3). Comparing the structure of the two compounds it would be possible that the bulky tri-fluor-methyl-group present in H479 hampers the interaction with the active site of most kinases.

The CKI protein kinase family is evolutionary conserved and ubiquitously expressed in eukaryotic organisms (*94*). In mammals 7 distinct isoforms (α, β, γ1, γ2, γ3, δ and ε) are expressed and members of this family are involved in many different physiological and cellular processes such as cell cycle progression, DNA damage repair, vesicular trafficking and cytokinesis (*88*). Some characteristic features of CK1 make it an especially interesting candidate for NS5A phosphorylation. CKI prefers acidic target sites and has a high preference for substrates containing phosphoserine or phosphothreonine within the consensus sequence pS/pTXXS*/T*, where pS and pT denote phosphorylated serine and threonine, S*/T* the phosphorylatable serine or threonine and X any amino acid (*95*). NS5A is an acidic protein with an isoelectric point around 5 and is heavily phosphorylated. In fact, NS5A contains at least 20 potential CKI phosphorylation sites. Interestingly, the region around the NS5A hyperphosphorylation sites is a hotspot for CKI recognition. As shown in Fig. 6, all identified serine residues important either for basal phosphorylation (S2194) or for hyperphosphorylation (S2197, S2201 and S2204) are placed within a potential CKI phosphorylation site.
To demonstrate that CKI is an important kinase for the formation of NS5A-p58 in cells, we inhibited NS5A hyperphosphorylation using known CK1 inhibitors. IC261 and SP600125 clearly reduced the formation of p58, whereas CKI-7 had only a slight effect, probably also due to poor ability to penetrate through membranes of living cells. One has to keep in mind, however, that these inhibitors affect other kinases as well at a similar concentration and therefore this type of experiments cannot be taken as a conclusive proof for the involvement of CKI.

The catalytic domain of CKI is highly conserved throughout different species and among different isoforms. This might explain why the NS5A-specific inhibitors work on the yeast CKI enzyme (Table 1), on the rat CKI-δ

isoform (Fig 18) as well as on the human CKI-α isoform (Fig. 21). However, the different CKI α, β, γ and δ isoforms have been shown to play important roles in distinct cellular pathways and therefore we aimed to identify the isoform(s) important for NS5A phosphorylation under physiological conditions using typical "gain-of-function" and "loss-of-function" experiments. Our results pointed to a special role of the α-isoform for NS5A hyperphosphorylation. While overexpression of the isoforms α and to a small extent also δ and ε increased NS5A hyperphosphorylation, RNA interference of only the α isoform was able to diminish the expression levels of NS5A-p58 (Fig. 20). The exceptions were the γ isoforms which did not seem to influence NS5A phosphorylation. These results indicate that all three isoforms are capable of recognizing the NS5A consensus sequence when ectopically overexpressed. However, we suggest that the physiologically relevant isoforms of CKI is the α-isoform. Two additional experiments support this result: i) rescue of the inhibited formation of p58 was only achieved upon overexpression of the α-isoform, but not upon expression of the δ- or ε-isoforms; ii) a clear indication whether a kinase is the target of a specific inhibitor is a change of EC50 dependent on the expression level of the kinase. We have shown that this correlation was confirmed only for the α-isoform.

With these results, one of the most interesting questions was whether the reduction of active CKI-α affects HCV replication. Inhibition of HCV replication upon incubation with the known CKI inhibitors could not be tested due to the high cytotoxicity of these compounds. We addressed this question by RNAi. Attenuation of CKI-α expression inhibited production of HCV RNA in cells containing actively replicating HCV subgenomes up to 60% after 5 days of CKI-α silencing. This result strongly supports a direct correlation between CKI-α expression and HCV replication. We also tried to activate replication of Con1 wild-type subgenomes in cells upon silencing of CKI-α, as described for the NS5A hyperphosphorylation inhibitors.

However, transfection of siRNAs and subgenomic RNA at different time points drastically increased cell mortality, and silencing efficiency might not be high enough for the establishment of replication. This type of experiment has to await the production of efficient small hairpin RNAs, which can be introduced into the cells by viral vectors.

All data obtained so far suggest that the CKI-α isoform is important for NS5A hyperphosphorylation.

So far, in vitro experiments demonstrated that NS5A is a substrate of several kinases however, a change of migration of NS5A in an SDS-gel was never

observed. We noticed here for the first time that E.coli expressed NS5A produces a slower migrating band upon incubation with CKI (fig 24). Even though we do not know whether the phosphorylated sites produced in vitro and in cells are the same, the similarity of the migration pattern is striking.
Exactly for this reason we refer to the two NS5A protein bands as p56* and p58*.
This result has two important implications: i. CKI-α is sufficient for basal phosphorylation of NS5A to produce (p56*), which is then converted to p58* by the same kinase. No other kinase is required for the production of both, p56* and p58* in vitro. This does obviously not exclude the possibility that in cells additional kinases might be involved in NS5A phosphorylation; ii. In contrast to what has been observed to date, no other nonstructural protein is required for the production of p58* in this in vitro experiment. It seems that protein folding and localization within the cell seem to play a crucial role for correct NS5A phosphorylation. NS5A has to be part of the replication complex and is localized together with all other HCV nonstructural proteins in the membranous structures through the N-terminal amphipathic helix. It would be possible that fixing the Nterminus of NS5A to the membranes puts constrains on protein folding which requires the presence of NS3/4A, NS4B and maybe also viral RNA for correct folding. On the other hand, removal of this constrain, that is attachment to the membranes via the amphipathic helix, does not facilitate NS5A hyperphosphorylation (Fig. 24C). This might indicate that localization of NS5A at the membranes is indeed important for hyperphosphorylation and one reason might be that CKI-α associates with membranes and its activity is differentially regulated by components within these membrane compartments (*94*).
The in vitro experiments using the NS5A peptides indicate that S2204 is a substrate residue for CKI-α. The requirement of pre-phosphorylated S2201 hints to a hierarchical order of phosphorylation. Even though S2201 is not a substrate of CKI-α in the context of a peptide, one cannot exclude the possibility that S2201 is phosphorylated by CKI-α in the context of a full-length protein. In fact, CKI is able to produce p58* in vitro, which might require pre-phosphorylation of S2201. In the more natural context of a living cell, this pre-phosphorylation of S2201 might well be performed by a different cellular kinase.
Many characteristics of CK1 α make it an interesting character in the role of NS5A-kinase. It exists as 4 different splicing variants, which vary by small insertions either within the catalytic domain (insert “L”) or close to the C-

terminus of the protein (insert "S"). These splice variants have been characterized biochemically and the most important difference resided in their subcellular localization: a variant containing the "L" insert was predominantly concentrated in the nucleus, while the CKI-α variant that lacks this insert was mostly cytoplasmic (*88*). In addition, the α-isoform has also been found to be associated with cellular membranes (*96*), vesicular structures (*97*) and is important for vesicle biogenesis (*98*). This is of particular interest because it has been demonstrated that all HCV nonstructural proteins are associated with intracellular membranes including the endoplasmic reticulum (ER) and Golgi (*99*), and that active replication most likely takes place in lipid rafts of the plasma membrane or internal membrane compartments such as the Golgi apparatus (*81*).
Unlike CKI-δ/ε, which are regulated by autophosphorylation of the C-terminal tail of the protein (*100*), CKI-α is constitutively active because it misses this regulatory domain and regulation of enzymatic activity has therefore to be achieved by other means. Subcellular localization and the hierarchical order of substrate phosphorylation are two possible explanations. Another interesting mechanism of activity regulation has been observed for the membrane-associated CKI-α isoform. This form is potently inhibited by phosphatidylinositol 4, 5-bisphosphate (PIP_2) (*101*). The presence of this inhibitory molecule varies between different membrane compartments due to different activity of the phosphatidylinositol 4-phosphate (PIP) 5-kinase required for the production of PIP_2 (*97*). Thus CKI-α is inhibited when associated with membranes containing PIP_2, whereas it is active when present in membranes without PIP_2. It is tempting to speculate that NS5A hyperphosphorylation varies according to the cellular compartment where it resides. The activity of membrane-bound CKI-α might be different in lipid rafts, where replication takes place, and at the cellular membrane, where virus assembly and/or virus exit is organized. Until recently this hypothesis was difficult to prove due to lack of a suitable infection system. Fortunately, an HCV strain has recently been isolated which is able to infect and replicate in cells in culture (*79*) and this system now offers the opportunity to study protein functions for replication as well as virus assembly and virus exit.

Hyperphosphorylation might have a number of structural and functional consequences for NS5A. Limited proteolysis of NS5A identified three relatively protease-resistant domains and two exposed protease-sensitive regions (*33*). Interestingly, the hyperphosphorylation region lies within one of these exposed protease-sensitive sequences and is therefore easily

accessible for regulatory proteins such as kinases. Recently the crystal structure of the N-terminal domain of NS5A has been published (*40*) and two interesting features were observed. First of all NS5A crystallized as a dimer and even though the stechiometry of NS5A within the replication complex is not known one could imagine that hyperphosphorylation changes the conformation of NS5A, which might provokes a switch between a monomeric and a dimeric state. Such kind of regulation has already been demonstrated for NSP5, a Rotavirus nonstructural protein phosphorylated by CKI (*102*). Secondly, the NS5A dimer forms a groove which could easily accommodate single- as well as double-stranded RNA. In fact, NS5A has been shown to bind RNA *in vitro* (*103*). Also in this case phosphorylation of the flexible linker region between the N-terminal and the C-terminal domains could change their relative position resulting in different capabilities to bind RNA. This latter case has been demonstrated for a protein substrate of CKI-α, which changes RNA-binding capacity upon phosphorylation by CKI-α (*104*). NS5A hyperphosphorylation could also be involved in a switch between translation of the plus-strand RNA and production of the minus-strand RNA by the NS5B polymerase (*105*).

The role of NS5A for viral replication and/or infection still remains a mystery. What becomes increasingly evident is that regulation of NS5A hyperphosphorylation plays an important role.

We started to investigate which of the cellular kinases are important for NS5A hyperphosphorylation, using small molecule inhibitors as well as genetic tools. Here we have identified the Casein Kinase I family of kinases as a possible target of our NS5A-specific kinase inhibitors and demonstrated that the CKI-α isoform is the kinase involved in NS5A hyperphosphorylation. We have shown in this work that several different protein kinases are targets of the NS5A hyperphosphorylation inhibitors, however, only CKI-α is important for NS5A hyperphosphorylation. The NS5A hyperphosphorylation inhibitors will have to be optimized in order to efficiently inhibit CKI-α but also to be as inefficient as possible against the other cellular targets. Using purified NS5A produced in E. coli plus CKI-α we were able to reproduce NS5A phosphorylation /hyperphosphorylation in vitro, a pattern normally only observed in eukaryotic cells in the context of the HCV polyprotein. Thus, identification of CKI-α as one of the cellular kinases important for NS5A phosphorylation/hyperphosphorylation is just a first piece within the complicated puzzle of NS5A phosphorylation. The detailed dissection of NS5A hyperphosphorylation might well facilitate the

understanding of the role of NS5A-p58 within the viral life-cycle of HCV and reveal novel therapeutic points of intervention.

4. *Materials and Methods*

4.1 Plasmids, antibodies and compounds

Plasmids were assembled by standard restriction digestion protocols or by PCR amplification of the sequence of interest with primers containing the desired mutations. The sequences of all plasmids were verified by automated sequencing using an ABI instrument.
Bla replicon constructs were derived from pHCVneo17.wt.
The *bla* coding region was PCR amplified by using pcDNA3-blaM (Aurora Biosciences) as the template with primers that introduced *Asc*I and *Pme*I sites at the 5' and 3' ends, respectively, and subcloned into the corresponding sites of pHCVneo17.wt. A silent mutation was subsequently introduced to eliminate a *Sca*I restriction site from the *bla* coding region.
The plasmid pHCV-AT, -SR, -K@2039 are identical to plasmid pHCVNeo17.wt described previously (*86*) but contains the mutation A2199T, S2204R and the inserction of a K in 2039 in NS5A. The plasmid pcD-BLA-wt contains the entire HCV sequence as described in plasmid wt-BLA (*89*) cloned into the pcDNA3 expression vector (Invitrogen). The coding regions for Casein Kinase I α, δ, ε and γ1 were taken from Image clones presented in the NCBI databank with the accession numbers BQ641640, BC015775, BC006490 and BC017236, respectively, and cloned into the EcoRV restriction site of the vector pcDNA3. The expression vector for CKI-γ2 and GSK3β were purchased from Invitrogen. The antibodies against CKI-α, CKI-δ and CKI-ε were purchased from Santa Cruz Biotechnology (Sc-6477 CK1α; Sc-6474 CK1δ specific; Sc-6471 CK1ε specific), anti- RSK1/RSK2/RSK3 (cell signalling 9347), JNK1 and 2 (Santa Cruz sc-1648, sc-7345), anti-RIP2 (cell signalling 4982) anti PKAC-α (cell signaling 4782), anti GAK (abcam ab22657), anti CAMKII (cell signaling 3362), anti MAPK p38 (cell signalling 9212).
The NS5A-specific antibodies and the NS5A-specific kinase inhibitors have been described in (*89*).
CKI-7 and was bought from US-Biological, SP 600125 and SB203580 were bought from SIGMA and IC261 was purchased from Calbiochem. The screening of the panel of kinases was performed by Upstate. All necessary information about the enzymes and the assay conditions can be obtained from the Manufacturer.

The Western Blots were performed using the corresponding secondary antibodies conjugated with Horse Raddish Peroxidase (HRP). The enzymatic reaction was developed using the ECL system (Amersham).

4.2 Cell culture

Cells highly competent for HCV replication (10A-IFN) were obtained by curing cells harboring neo replicons. The cells were seeded at 80% confluence and then cultured at high cell density in the presence of 100 U of IFN-α/ml for 11 days. 10A-IFN were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. For cells supporting subgenomic replicons, 0.8 mg of G418 (Geneticin; Gibco-BRL)/ml was added.

4.3 Selection of NS5A phosphorylation inhibitors (in vitro assay).

Huh7 cells containing the Con1 subgenomic replicon with the adaptive mutation A2199T (Huh7-68) were grown to 50% confluence, and the protein extract was prepared as follows. Cells from a 15-cm-diameter dish were lysed with 500 µl of NETN buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with 5 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride, 100 mM NaF, 20% glycerol, and the protease inhibitor cocktail Complete (Roche) as described previously (*60*). For immunoprecipitation, 2.5 µl of NS5A-specific antibody (*70*) was bound to 12 µl of protein A-Sepharose in 300 µl of NETN for 1 h at 4°C. After the incubation, protein A-Sepharose was washed once with NETN and 30 µg of total protein was immunoprecipitated in 300 µl of NETN for 1 h at 4°C. After binding, the resin was washed once with NETN supplemented with 5 mM DTT and once with kinase buffer (50 mM Tris-HCl [pH 7.5], 5 mM MnCl, 5 mM DTT, and 50 mM NaF). For the in vitro kinase reaction, 50 µl of kinase buffer supplemented with 2.5 µCi of [γ-33P]ATP and 2.5 µl of either 100 µM compound dissolved in dimethyl sulfoxide (DMSO; final concentration, 5 µM) or DMSO alone was added to the immunoprecipitate. Reaction mixtures were incubated for 45 min at 37°C, and the reaction was terminated by the addition of 25 µl of 3x protein sample buffer. Proteins were loaded onto a sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis (PAGE) gel and autoradiographed.).

4.4 Analysis of resistant clones

Total RNA was extracted using a RNeasy® mini kit (Invitrogen) and the NS5A cDNA was generated by RT-PCR of total RNA with SuperScript II (Invitrogen), according to the manufacturer's instruction. We mixed 9µl of RNA with 1µl of oligo HCVG34 (5 µM) [ACATGATCTGCAGAGAGGCCAGT]. The amplification was performed with Finnzymes using oligo HCVG205 [CCCCGTTCATCGATTGGGGAGTAGATAG] and HCVG206 [GCTGCATCATCACTAGTCTCACAGGCC]
To DNA sequence was evaluated for the 5' and 3' cDNA region by an automatic sequence.

4.5 Cell-enzyme-linked immunosorbent assay (ELISA)

Cells were monitored for expression of the NS3 protein by enzyme-linked immunosorbent assay (ELISA) with the anti-NS3 like is described by Trozzi et al (27).

4.6 Transient expression of HCV proteins and preparation of labelled extracts.

3.5 x 10^5 10A-IFN cells / 35-mm diameter dish were plated the day before the experiment. The cells were infected with vaccinia virus vTF7-3 for 1 h at 37°C and then, if not mentioned different in the figure legend, 2 µg of total plasmid DNA was transfected using Fugene6 (Roche) as transfection reagent.. After 4 h of transfection, cells were starved for 1 h in minimal essential medium without methionine (GibcoBRL) and labelled for 3 h with 100 µCi of 35S-labelled methionine (Promix; Amersham) per ml. For [32P]orthophosphate labelling, cells were washed once after transfection with Dulbecco's modified Eagle's medium without phosphate (ICN) and labelled for 4 h in the same medium containing 500 µCi of [32P]orthophosphate (285.5 Ci/mg; NEN) per ml. Cells were harvested, and cell extract was prepared in 150 µl of lysis buffer (25 mM sodium phosphate [pH 7.5], 20% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride).

To assess the inhibitory activity of the compounds, cells were incubated with 5 µM concentrations of the indicated compounds during starvation and the compounds were present during the whole time of labeling.

4.7 Immunoprecipitation.

For immunoprecipitation under denaturing conditions, 20 µl of extract was heated at 95°C for 4 min in the presence of 1% sodium dodecyl sulfate (SDS) and 10 mM DTT. Five microliters of HCV-specific antisera was incubated with 50 µl of protein A-Sepharose (PAS) for 1 h at 4°C in 300 µl of immunoprecipitation buffer (IPB150; 20 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Triton X-100), washed once with IPB150, and incubated with the extract for 1 h at 4°C in a volume of 500 µl of IPB150. All subsequent procedures were as described in (*106*). Protein was detached from the PAS-resin by boiling in SDS sample dye.

4.8 Assays for bla reporter activity.

Medium was removed, and cells were stained for 90 min with CCF4-AM (Aurora Biosciences Corp.) in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, pH 8.0. For quantitation of the fraction of cells harboring bla replicons, cells were photographed by using a digital charge-coupled device color camera and green and blue cells were counted by DIP with Image-Pro Plus software. Alternatively, fluorescence was measured by using a CytoFluor 4000 fluorescence plate reader.

4.9 RNA interference

siRNAs were transfected by electroporation. The protocol used for electroporation was already described previously(*89*). Briefly, 10 µM of siRNA were electroporated in $1x10^6$ 10A-IFN cells in a volume of 0.1 ml. After electroporation, 4.5 x 10^5 cells were plated in a 35-mm-diameter dish and incubated for 2 days. Protein expression with the Vaccinia infection/transfection system was performed as described previously (Neddermann 1999). Double-stranded siRNAs were annealed *in vitro* before usage.

The siRNA sequences are described below:

Cit sense: 5'-accuuuaucuggucauggatt-3'; GAK sense: 5'-gacaagaugcgggacuuuatt-3'; RIPK2 sense: 5'-ggaaucauguggauccucutt-3'; PKA sense: 5'-cagaguuccuugcaucuaauutt-3'; RSK2 sense: 5'-cucaugacuccuuguuacauutt-3'; CaMKIIδ sense: 5'-cacuacuugguguuugauuuutt-3';

Quantitative PCR was performed as described (21) using 100 ng of RNA.

Primers and probes are described below Cit for: 5'-cag ggttgggaagtccttagg-3'; Cit rev: 5'-gttctgtaccagttgccaggaat-3'; Cit probe: 5'-ctgcggaccctcctattagagtacg-3'; GAK for: 5'-cccgaaggaacagctgattc-3'; GAK rev: 5'-cgcccgaaacaccaaataaa-3';GAK probe: 5'-cctcccgcaattgtcacgtctgtg-3'; RIPK2 for: 5'-acgtctgcagcctggtatagc-3'; RIPK2 rev: 5'-ggcaggcttctgtcatttgg-3'; RIPK2 probe: 5'-tggatccagagcaaaagggaagacattgt-3'; PKA for: 5'-agccggagaatctgctcattg-3'; PKA rev: 5'-cacgcgcttggcgaaac-3'; PKA probe: 5'-ccagcagggctacattcaggtgacagactt-3';RSK2 for: 5'-cttgtgtgtgtgaaaagtcagttatga-3'; RSK2 rev: 5'-gtacactgcatgtgaaatgtggaa-3'; RSK2 probe: 5'-tctggagagaagctgtggtgtgtatgctgt-3'; CaMKIIδ for: 5'-gcggatcgtttcgcaact-3'; CaMKIIδ rev: 5'-gccgggaaatggaaaaaca-3';

CaMKIIδ probe: 5'-ttgccactcgtcccgtgcctg-3'; All probes contain the 6FAM dye at the 5'-end and the TAMRA quencher at its 3'-end. As endogenous standard we used the GAPDH probe, containing the VIC-dye at its 5'-end (Applied Biosystems). Reactions are conducted in three stages under the following conditions: stage 1, 30 min at 48°C ; stage 2, 10 min. at 95°C, stage 3, 15 sec at 95°C and 1 min. at 60°C, 40 cycles. The total volume of the reaction is 50 µl.

4.10 Inhibition of HCV replication

In order to measure inhibition of HCV replication, Huh7 cells stably expressing a HCV subgenomic replicon containing the adaptive mutation S2204R (SR3) were used. Selection of clones was performed as described previously (Lohmann et al., 1999), except that 10A-IFN cells instead of naïve Huh7 cells were used. siRNAs were electroporated in SR3 as described above. After electroporation, cells were plated in a 12-well plate ($2x10^5$ cells for day 2, $1.5x10^5$ cells for day 3, $1x10^5$ cells for day 4 and $8x10^4$ cells for day 6). Quantitative PCR was performed as described (Neddermann et al., 2004). Briefly, 10 ng of RNA (HCV) or 100 ng (kinases) were used for the reaction. Primers and probes are described in Supplementary Information.

4.11 Immobilization of L782479 and L775852s

2 ml of drained epoxy-activated Sepharose 6B (1g freeze-dried powder gives 3.5 ml final volumes of gel) was washed using distilled waters and than with coupling buffer (3x) (50% dimethylformamide/0.1 M Na2CO3). 10mM of L782479 and 5 mM of L852 were dissolved in coupling buffer (1 ml final) and were added to the gel. Coupling was performed overnight at 37 °C in the dark. After three washes with 50% dimethylformamide/0.1 M Na2CO3, remaining reactive groups were blocked with 1 M ethanolamine, pH 11. Subsequent washing steps were performed according to the manufacturer‘s instructions. To generate the control matrix (Ctrl), epoxy-activated Sepharose 6B was directly reacted with 1 M ethanolamine pH 11 and equally treated as described above. The matrices were stored at 4 °C in the dark.

4.12 Cell Lysis and in Vitro Association Experiments

10A-IFN cells were lysed in Triton X-100 lysis buffer (TL buffer) containing 50 mM HEPES, pH 7.5, 500 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 10 mM EGTA, 10% glycerol plus additives (10 mM sodium fluoride, 1 mM orthovanadate, 1mM dithiothreitol, protease inhibitors cocktail complete). For analytical in vitro association experiments, lysates were precleared by centrifugation and equilibrated to 1 M NaCl. Then 1 ml of the high salt lysate (2mg/ml) was incubated together with 100 µl of

drained inhibitors-matrix for 1 h at 4 °C. Afterward, the matrices were washed with 30V of TL-buffer plus 1 M NaCl and 30 CV with TL-buffer low salt (20mM NaCl); both steps were performed without additives. Bound proteins were eluted with 2.5 V TL buffer-low salts containing neither 100mM Mg-ATP or 100 mM Mg-ATP+1mM compound or only 1 mM of bought compounds. After elution, the resin was eluted by boiling of the affinity beads in 1.5x SDS sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and immunoblotted with the corresponding antibodies. The preparative binding experiments using 88 x 106 cells, 1ml of inhibitor-gel, mass spectrometric analysis were carried and the elution was performed as we described before.

4.13 In gel digestion

After silver staining the gel, bands of interest were excised, and the gel pieces are placed in a eppendorf. The gel pieces are sequentially washed with 100µl of 50% Acetonitrile in water (5 min.), 100µl of Acetonitrile (5 min.), 100µl of 0.1M NH4HCO3 (5 min.), 100µl of Acetonitrile (5 min.) and finally incubated for 45 minutes at 4°C in the presence of 12.5ng/µl Trypsin in 50mM NH4HCO3. The trypsin buffer was then replaced with 50mM NH4HCO3 (without trypsin) and the gel pieces are incubated overnight at 37°C.

4.14 Mass spectrometry and database search

After overnight digestion the supernatant is recovered in a 96-well V-bottom plate and the remaining tryptic peptides are further extracted from the gel pieces by incubation in 50µl of 20% Formic Acid in water (15min). The supernatants were pooled together and dried down in a SpeedVac concentrator. Peptides are finally resuspended in 0.5% Acetic Acid and subjected to C_{18} ZipTip (Millipore) desalting prior to Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI–TOF–MS) or to capillary Liquid Chromatography coupled to Electrospray Ion Trap Tandem Mass Spectrometry (cLC–ESI–IT–MS–MS) for protein identification. Peptide mass fingerprint experiments (MALDI-TOF-MS) are performed using a Voyager sSTR mass spectrometer (Applera, USA) MASCOT software package is used to identify proteins from independent non-redundant sequence databases. Collision-induced dissociation (CID)-

derived information are obtained using a LCQ Deca XpPlus mass spectrometer (ThermoFinnigan, USA) equipped with an micro-electrospray source connected to a in-house packed C18 column (100 mm × 0.10 mm). Data were elaborated using the TurboSequest software provided by the manufacturer. Candidates with Xcorr values >2.5 were considered identified with high confidence.

4.15 Electrophoresis and Western Blotting

Following heat denaturation for 5 min, the proteins bound to the kinase inhibitors-matrix were separated by 10% SDS-PAGE followed by immunoblotting analysis or silver staining. For immunoblotting, proteins were transferred to 0.45-µm nitrocellulose filters (Schleicher and Schuell). These were blocked with 5% low fat milk in Tris-buffered saline/Tween 20, incubated for 1 h with antibodies, and analyzed by Enhanced Chemiluminescence (ECL, Amersham).

4.16 In Vitro Kinase Assays

All protein kinase activities were linear with respect to time in every incubation. Assays were performed for 30 min at ambient temperature in 50 or 25 µl incubations using [γ-^{33}P] ATP, . The concentrations of ATP and magnesium acetate were 0.125 mM and 10 mM respectively, unless stated otherwise. This concentration of ATP is 5–10-fold higher than the K_m for ATP of most of the protein kinases studied in the present paper, but lower than the normal intracellular concentration, which is in the millimolar range. All assays were initiated with ATP. Manual assays were terminated by the addition of 5 µl of 0.5 M phosphoric acid before spotting aliquots on to P81 filter mats (Wallac). All papers were then washed four times in 75 mM phosphoric acid to remove ATP, once in methanol and then dried and counted for radioactivity.
MAPKAP-K1b/RSK2, PKA, were assayed in 8 mM Mops, pH 7.0, containing 0.2 mM EDTA. Substrate peptides (single-letter code for amino acids) were: MAPKAP-K1b/RSK2, KKLNRTLSVA (30 μM); PKA, LRRASLG (30 μM); SAPK2a/p38, was assayed in 25 mM Tris/HCl, pH 7.5, containing 0.1 mM EGTA, with myelin basic protein (0.33 mg/ml) as substrate. MAPKAP-K2 and PRAK were assayed in 50 mM sodium β-glycerophosphate, pH 7.5, containing 0.1 mM EGTA, with the peptides

KKLNRTLSVA (30 μM) and KKLRRTLSVA (30 μM) respectively as substrate. CAM-KII was assayed in 50 mM Hepes, pH 7.4, containing 5 mM $CaCl_2$ and 0.03 mg/ml calmodulin, with the peptide KKLNRTLSVA (60 μM) as substrate. JNK1,JNK2 were assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1% 2-mercaptoethanol with the following peptides: 3 μM GST–ATF2-(19–96) (where ATF2 is activating transcription factor 2).

4.16 Expression and purification of NS5A and CKI-α

Expression of NS5A from plasmid pET26Ub-His-delta32 or pET26Ub-Hisdelta32-SR and purification was performed as described (*107*). Isolation of recombinant Baculovirus (Bacmid) expressing Flag-CKI-α was performed as described by manufacturer (BAC-TO-BAC Baculovirus expression System; GIBCO BRL). Protein expression was performed as described previously for NS5A (*67*) with the exception that infection was performed at room temperature.
Cells extract was prepared in lysis buffer containing 25 mM HEPES pH 7.5, 300mM NaCl, 1 % Triton X-100, 40 % glycerol, 10mM NaF, 1 mM EDTA, 2mM DTT,protease inhibitors (Complete, Roche). The cell extract was clarified by centrifugation and glycerol concentration was diluted to 20 % before incubation with anti-Flag agarose (Sigma-Aldrich) for 4 hours at 4°C. The anti-Flag agarose was washed extensively with lysis buffer without glycerol. Elution of the FLAGtagged proteins was performed with 3 volumes of elution buffer (50mM HEPES pH 7.5, 100 mM NaCl, 5% glycerol, 0.05% Triton X-100) containing 100 µg / ml of FLAG-peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, Sigma-Aldrich) for 1 hour at room temperature. Eluted proteins were stored at −20 °C.

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