

Hepatitis C Virus NS5A Is a Direct Substrate of Casein Kinase I- α , a Cellular Kinase Identified by Inhibitor Affinity Chromatography Using Specific NS5A Hyperphosphorylation Inhibitors*

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The hepatitis C virus encodes a single polyprotein that is processed by host and viral proteases to yield at least 10 mature viral proteins. The nonstructural (NS) protein 5A is a phosphoprotein, and experimental data indicate that the phosphorylation state of NS5A is important for the outcome of viral RNA replication. We were able to identify kinase inhibitors that specifically inhibit the formation of the hyperphosphorylated form of NS5A (p58) in cells. These kinase inhibitors were used for inhibitor affinity chromatography in order to identify the cellular targets of these compounds. The kinases casein kinase I (CKI), p38 MAPK, CIT (Citron Rho-interacting kinase), GAK, JNK2, PKA, RSK1/2, and RIPK2 were identified in the high affinity binding fractions of two NS5A hyperphosphorylation inhibitors (NS5A-p58-i). Even though these kinases are targets of the NS5A-p58-i, the only kinase showing an effect on NS5A hyperphosphorylation was confirmed to be CKI- α . Although this finding does not exclude the possibility that other kinase(s) might be involved in basal or regulatory phosphorylation of NS5A, we show here that NS5A is a direct substrate of CKI- α . Moreover, *in vitro* phosphorylation of NS5A by CKI- α resulted for the first time in the production of basal and hyperphosphorylated forms resembling those produced in cells. *In vitro* kinase reactions performed with NS5A peptides show that Ser-2204 is a preferred substrate residue for CKI- α after pre-phosphorylation of Ser-2201.

The hepatitis C virus (HCV)³ is a member of the Flaviviridae family of single-stranded, positive sense RNA viruses and the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide. Intensive studies since the discovery of the virus in 1989 have improved therapeutic options, but they are still inadequate for the majority of patients.

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³ The abbreviations used are: HCV, hepatitis C virus; NS, nonstructural; PKA, cAMP-dependent protein kinase; CKI, casein kinase I; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; CaMK, calcium/calmodulin-dependent protein kinase; RIPK2, receptor-interacting serine/threonine protein kinase 2; siRNA, short interfering RNA; MOPS, 4-morpholinepropanesulfonic acid.

The 9.6-kb RNA genome of HCV is translated into a single polyprotein, which is then co- and post-translationally processed by host and viral proteases to yield at least 10 mature viral proteins (C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) (1).

We focused our interest on the nonstructural protein NS5A. NS5A, together with all other nonstructural proteins, is associated with membranous structures thought to be the scaffold for the formation of the viral replication complex (2, 3). However, although NS2, NS3/4A, NS4B, and NS5B are tightly associated with membranes because of the presence of membrane-anchoring or transmembrane domains (4), NS5A associates with the endoplasmic reticulum by an amphipathic α -helix (5). This might result in a less tight interaction with membranes, and dissociation of NS5A from the replication complex might be facilitated. In support of this hypothesis are recent publications that demonstrate that mutations within NS5A can be trans-complemented, whereas trans-complementation for all other nonstructural proteins failed (6, 7).

This flexibility might facilitate the multiple functions attributed to NS5A. NS5A has been described to influence many different cellular pathways (8). However, direct interaction with the viral RNA-dependent RNA polymerase (9) as well as with RNA (10) support the fact that NS5A also plays an important role in viral replication.

Considering the numerous functions ascribed to this single protein, it is not surprising that post-translational events play a crucial role for the regulation of functions of NS5A. It has been demonstrated by several groups that NS5A is a target of different cellular proteases (11, 12), and it is easy to imagine that different cleavage products might exert different cellular functions. However, the most studied post-translational modification of NS5A identified to date is phosphorylation. NS5A is expressed as a basally phosphorylated (p56) and a hyperphosphorylated (p58) form (13), the production of the latter form requires the presence of other HCV nonstructural proteins (14–16). The requirements for NS5A hyperphosphorylation are in remarkable agreement with two other previous observations as follows: trans-complementation could be obtained only when NS5A was expressed in the context of an intact polyprotein coding from NS3 to NS5A (6), and wild type NS5A exerted a trans-dominant negative effect on adapted subgenomic replicons only when expressed in the same context (17). Thus it seems that the presence of the NS3, NS4A, and NS4B proteins

are required for the production of a correctly folded, functional NS5A protein. Whether NS5A hyperphosphorylation is the cause or the consequence of correct folding in this context is still to be established.

Besides the presence of other nonstructural proteins, NS5A hyperphosphorylation requires the presence of three serine residues (18) that are situated in a region between the first and the second N-terminal domains of NS5A (19). Even though the functional relevance of the different phosphorylated forms is still unknown, it has become more and more evident that a tight regulation of NS5A phosphorylation is important for the outcome of HCV RNA replication. The Con1 wild type sequence is able to replicate in cells only after acquisition of adaptive mutations, which in most cases reduce NS5A hyperphosphorylation (20), or after reduction of hyperphosphorylated NS5A upon incubation with NS5A hyperphosphorylation-specific kinase inhibitors (21). In this case, the alteration of NS5A phosphorylation increases replication efficiency. There are, however, other examples that indicate that alterations of a functional ratio between NS5A-p56 and p58 negatively influence the outcome of productive replication/infection. An actively replicating HCV subgenome expressing a reduced level of NS5A-p58 is completely inhibited if hyperphosphorylation is further decreased either by introduction of a second adaptive mutation (22) or by the addition of the NS5A hyperphosphorylation inhibitors (21). In addition, introduction of a mutation that reduces NS5A hyperphosphorylation into an infective clone abrogates infection in the chimpanzee model (23). The latter example, in particular, underlines the importance of NS5A-p58 for replication/infection and points to the fact that the cellular kinase responsible for NS5A hyperphosphorylation might be a good alternative antiviral target besides the viral enzymatic targets.

We aimed to identify this/these cellular kinase(s) using the specific NS5A hyperphosphorylation inhibitors (NS5A-p58-i). Upon screening of a panel of cellular kinases *in vitro*, we identified the family of casein kinase I (CKI) as a target of the compounds, and using genetic tools we confirmed that the α -isoform of CKI is important for the formation of NS5A-p58 (24). Although this strategy helped us to identify CKI- α , it has several shortcomings because only a small subset of the cellular kinases could be tested (60 out of >500). Because of this limitation, we cannot exclude the possibility that other cellular kinases not present in the previous screen are a target of the NS5A hyperphosphorylation inhibitors and might participate on the same pathway finally resulting in NS5A hyperphosphorylation. We therefore adopted an alternative methodology, the inhibitor affinity chromatography, to confirm CKI- α as a target of the compounds in a more physiological context in the presence of ATP-binding proteins and competing cellular kinases and to eventually identify additional kinases that might contribute to NS5A hyperphosphorylation.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, Enzymes, and Peptides—The antibodies against CKI- α , CKI- δ , CKI- ϵ , and JNK2 were purchased from Santa Cruz Biotechnology and against p38 MAPK, RSK, and PKA from Cell Signaling Technology. The NS5A-specific

antibodies have been described previously (21). All NS5A peptides were synthesized by Bio-Synthesis Inc. CKI- δ and its substrate peptide were purchased from New England Biolabs. CKII, p38 MAPK, PKA, JNK1, JNK2, RSK2, and their respective substrates were obtained from Upstate. The plasmids pcD-BLA-wt and pET26Ub-His- Δ 32 have been described previously (21, 25). Plasmid pET14-R30-SR-K4 contains the coding region of Con1-NS5A with the following modifications. The first 29 amino acids were deleted, and the NS5A coding region starts with Arg-30. Ser-232 was mutated to arginine (amino acid of the HCV polyprotein S2204R), and four lysine residues were added to the C terminus to increase solubility of the protein. The DNA plasmid pHCVNeo-SR is identical to pHCVNeo17.wt, as described previously (26), but contains the mutation S2204R in NS5A. Plasmid pBAC-FLAG-CKI- α contains the CKI- α coding region with an N-terminal FLAG tag. The coding region of CKI- α with the N-terminal FLAG tag was taken from plasmid pCD-FLAG-CKI- α , as described previously (24), and cloned into the BamHI/NotI cloning sites of pBAC-1 (Novagen). All modifications were performed using standard recombinant DNA technology. Compounds were synthesized as described previously (27).

Immobilization of H479 and A852-m—2 ml of drained epoxy-activated Sepharose 6B (GE Healthcare) were equilibrated with coupling buffer (50% dimethylformamide, 0.1 M Na₂CO₃), and 4 ml of 10 mM H479 or 5 mM of A852-m, dissolved in coupling buffer, were incubated overnight at 37 °C in the dark with the resin. After the incubation the resin was washed with coupling buffer, and the remaining reactive groups were blocked with 1 M ethanolamine in coupling buffer for 5 h at 37 °C. Subsequent washing steps were performed according to the manufacturer's instructions. To generate the control matrix, epoxy-activated Sepharose 6B was directly reacted with 1 M ethanolamine in coupling buffer and equally treated as described above. The matrices were stored at 4 °C in the dark.

Extract Preparation and Inhibitor Affinity Chromatography—10A-IFN cells (26) 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, 1 mM dithiothreitol, protease inhibitors mixture Complete (Roche Applied Science)). For protein binding in batch, lysates were pre-cleared by centrifugation and equilibrated to 1 M NaCl. Cell lysate (2 mg of total protein) was incubated with 100 μ l of drained inhibitor-matrix for 2 h at 4 °C with or without 2 mM of compound as indicated in the figure legends. Unbound proteins were separated by centrifugation, and resin was washed extensively first with TL buffer, 1 M NaCl and then with TL buffer, 20 mM NaCl. Bound proteins were directly denatured by addition of 1 volume of 2 \times Laemmli sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the corresponding antibodies. Secondary antibodies conjugated with horseradish peroxidase were used, and enzymatic reactions were developed using the ECL system (GE Healthcare). For preparative binding experiments, protein extract was prepared from 9×10^7 10A-IFN cells as described above, equilibrated to 1 M NaCl, and loaded directly onto an inhibitor-matrix contain-

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ing chromatographic column. After binding, the column was washed with 30 volumes of TL buffer, 1 M NaCl and 30 volumes of TL buffer, 20 mM NaCl. Bound proteins were first eluted with 3 volumes TL buffer, 20 mM NaCl containing 100 mM Mg-ATP and subsequently with 3 volumes of TL buffer, 20 mM NaCl containing 100 mM Mg-ATP plus 1 mM compound.

Mass Spectrometry—Eluted proteins (400 μ l) were concentrated with the clean-up kit (GE Healthcare), separated by SDS-PAGE and visualized by silver staining. The bands of interest were excised and digested with trypsin for mass spectrometry-based protein identification. Briefly, the gel pieces were incubated with 20 μ l of 12.5 ng/ μ l trypsin sequencing grade (Promega, Madison, WI) in 50 mM NH_4HCO_3 for 45 min at 4 °C. Trypsin was then replaced with 50 mM NH_4HCO_3 , and the gel pieces were left overnight at 37 °C. Tryptic peptides were extracted from the gel pieces by incubation in 50 μ l of 20% formic acid in water (15 min) and pooled together with the supernatants. The tryptic peptides were dried down in a Speed-Vac concentrator, resuspended in 0.5% acetic acid, and analyzed by liquid chromatography coupled to electrospray ion trap tandem mass spectrometry or subjected to ZipTip μ -C18 (Millipore) desalting prior to matrix-assisted laser desorption/ionization time of flight mass spectrometry. For liquid chromatography coupled to electrospray ion trap tandem mass spectrometry experiments, an LCQ Deca XP-Plus mass spectrometer (Thermo Electron, San José, CA) equipped with an in-house built micro-electrospray source connected to an in-house packed C18 column (100 mm \times 0.10 mm, 5 μ m particle size) was used. Protein identification from collision-induced dissociation data was done with TurboSequest software provided by the manufacturer.

For matrix-assisted laser desorption/ionization time of flight mass spectrometry experiments, a Voyager-STR mass spectrometer (Applied Biosystems, Foster City, CA) was used, which operated in positive reflectron mode using α -cyano-4-hydroxycinnamic acid (10 mg/ml in water/acetonitrile 3:7, 1% trifluoroacetic acid) as matrix. Internal calibration was used. MASCOT software package (Matrix Science, London, UK) was used to identify proteins from independent nonredundant sequence data bases.

Protein Expression and Metabolic Labeling of Proteins—Protein expression using the vaccinia T7 infection/transfection system was performed as described (14). 3.5×10^5 10A-IFN cells/35-mm diameter dish were plated the day before the experiment. 2 μ g of total plasmid DNA was transfected using FuGENE 6 (Roche Applied Science) as transfection reagent. Metabolic labeling and immunoprecipitation were performed as described. Inhibitors were added during the starvation reaction and were present during metabolic labeling of the cells.

RNA Interference—siRNAs were transfected by electroporation (21). Briefly, 10 μ M siRNA were electroporated in 1×10^6 10A-IFN cells in a volume of 0.1 ml. After electroporation, 4.5×10^5 cells were plated in a 35-mm diameter dish and incubated for 2 days. After 2 days, HCV proteins were expressed with the vaccinia T7 infection/transfection system as described above. The siRNA sequences are described as follows: CIT sense, 5'-accuuuauccugguauccaggatt-3'; GAK sense, 5'-gacaagau-gcgggacuuuatt-3'; RIPK2 sense, 5'-ggaucauguggauccucutt-3';

PKA sense, 5'-cagaguuccuugcaucuaauutt-3'; RSK2 sense, 5'-cucaugacuccuuguuacauutt-3'; CaMKII δ sense, 5'-cacuacuugg-uguuugauuuutt-3'. siRNAs for CKI- α , CKI- δ , and CKI- ϵ were described previously (24).

Quantitative PCR was performed as described (21) using 100 ng of RNA. Primers and probes are described as follows: CIT forward, 5'-cagggttggaagtccttagg-3', CIT reverse, 5'-gttctgtaccagttgccaggaat-3', and CIT probe, 5'-ctgcggacaccttattagatgacg-3'; GAK forward, 5'-cccgaaggaacagctgattc-3', GAK reverse, 5'-cgccccgaaacaccaaataaa-3', and GAK probe, 5'-cctcccgcaattgtcagctctgtg-3'; RIPK2 forward, 5'-acgtctgcagcctggatagc-3', RIPK2 reverse, 5'-ggcaggcttctgtcatttgg-3', and RIPK2 probe, 5'-tggatccagagcaaaagggagacattg-3'; PKA forward, 5'-agccggagaatctctcattg-3', PKA reverse 5'-cacgcgcttgccgaaac-3', and PKA probe, 5'-ccagcagggtacattcaggtgacagactt-3'; RSK2 forward, 5'-cttctgtgtgtgaaagtcagttatga-3', RSK2 reverse, 5'-gtacctgcatgtgaaatgtggaa-3', and RSK2 probe, 5'-tctggagaaagctgtggtgtgtatgctgt-3'; and CaMKII δ forward, 5'-gcgatcgtt-tcgcaact-3', CaMKII δ reverse, 5'-gccgggaaatggaaaaaca-3', and CaMKII δ probe, 5'-ttgccactgtcccgtgctg-3'. All probes contain the 6-carboxyfluorescein dye at the 5'-end and the 6-carboxy-*N,N,N',N'*-tetramethylrhodamine TAMRA quencher at its 3'-end. As endogenous standard we used the glyceraldehyde-3-phosphate dehydrogenase 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 s at 95 °C and 1 min at 60 °C, 40 cycles. The total volume of the reaction is 50 μ l.

In Vitro Kinase Assays—All protein kinase activities were linear with respect to time in each incubation. If not mentioned differently, all reactions were performed in the presence of 125 μ M cold ATP, 0.2 μ l of [γ - ^{33}P]ATP (2 μ Ci/point), and 18 mM MgCl_2 in reaction buffer (10 mM MOPS, pH 7.2, 12.5 mM β -glycerol phosphate, 2.5 mM EDTA, 0.5 mM sodium orthovanadate, and 0.5 mM dithiothreitol) in a final volume of 50 μ l for 30 min at room temperature. For the calculation of IC_{50} values, compounds were added in a range between 0.1 nM and 1 mM resulting in a final Me_2SO concentration of 5%. The reactions were initiated by the addition of ATP and stopped by the addition of 10 μ l of 3% phosphoric acid, and 20 μ l of the reaction were spotted onto P30 Filtermat (Wallac). The filter was washed three times for 5 min with 75 mM phosphoric acid and once with methanol. Radioactivity was counted in 5 ml of Ready Protein scintillation mixture (Beckman Coulter). CKI- δ , CKI- α (4 μ l of purified fraction, see below), and CKII were tested as described previously (24). PKA (5 ng) was assayed in the presence of the PKC/CaMK inhibitor mixture (Upstate) using 30 μ M peptide substrate, and RSK2 (10 ng) was tested using 100 μ M peptide substrate. Activity of p38 MAPK (80 ng) was assayed using myelin basic protein (40 μ g) as substrate. Activity of JNK1 and JNK2 (30 ng) was assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol using 3 μ M ATF2 as substrate peptide. For *in vitro* phosphorylation of NS5A, 2 μ M purified NS5A (25) was incubated in the same conditions as described above in a final volume of 60 μ l with 12 μ l of purified CKI- α (see below), 120 ng of CKI- δ , 250 ng of CKII, or 500 ng of PKA, respectively, for 1 h at room temperature.

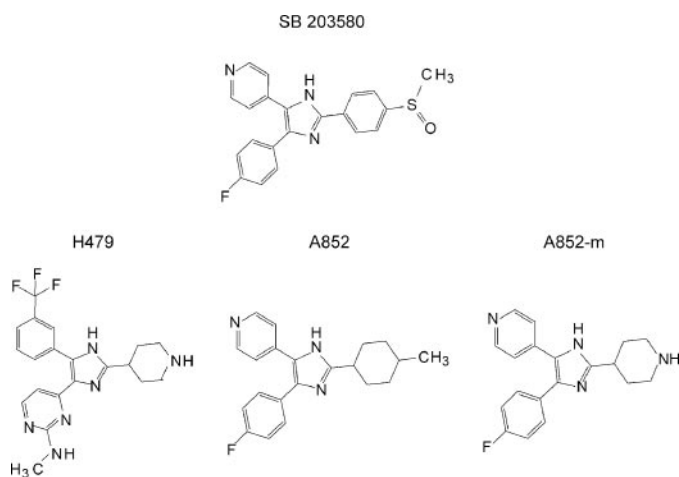


FIGURE 1. Chemical structure of the kinase inhibitors used in this work.

Expression and Purification of NS5A and CKI- α —Expression and purification of NS5A from plasmid pET26Ub-His- Δ 32 was performed as described (25). Expression of NS5A from plasmid pET14-R30-SR-K4 was performed in BL21-codon plus (Stratagene). Cells were grown in LB medium to a density of 0.8 and induced with 0.4 mM isopropyl 1-thio- β -D-galactopyranoside at 18 °C for 16 h. Purification was performed as described (28) with the following modifications. All buffers contained 0.3% *n*-octyl- β -D-glucopyranoside instead of Triton X-100. Lysis buffer contained 25 mM Hepes/HCl (pH 7.5), 50% glycerol, 0.3% *n*-octyl- β -D-glucopyranoside, 500 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, protease inhibitors (Complete, Roche Applied Science). Gel filtration was performed in 200 mM NaCl. Active fractions were loaded onto a Mono S column after gel filtration for concentration.

Isolation of recombinant baculovirus (Bacmid) expressing FLAG-CKI- α was performed as described by manufacturer (Bac-to-Bac[®] baculovirus expression system; Invitrogen). Protein expression was performed as described previously for NS5A (28) with the exception that infection was performed at room temperature. Cell extract was prepared in lysis buffer containing 25 mM HEPES pH 7.5, 300 mM NaCl, 1% Triton X-100, 40% glycerol, 10 mM NaF, 1 mM EDTA, 2 mM dithiothreitol, protease inhibitors (Complete, Roche Applied Science). The cell extract was clarified by centrifugation and glycerol concentration was diluted to 20% before incubation with anti-FLAG-agarose (Sigma) for 4 h at 4 °C. The anti-FLAG-agarose was washed extensively with lysis buffer without glycerol. Elution of the FLAG-tagged proteins was performed with 3 volumes of elution buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 5% glycerol, 0.05% Triton X-100) containing 100 μ g/ml of FLAG-peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys; Sigma) for 1 h at room temperature. Eluted proteins were stored at -20 °C.

RESULTS

Design of Inhibitor Affinity Chromatography—We have previously identified specific inhibitors of the NS5A hyperphosphorylation (21). The chemical structure of two of these compounds, H479 and A852, is shown in Fig. 1. The compounds belong to the class of 2, 4, 5-trisubstituted imidazoles, which are known to bind to the active site of kinases and are competitive

with ATP. To purify the kinases targeted by these compounds, we generated affinity resins by immobilizing the kinase inhibitors to a solid matrix. 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 that can be attached to the matrix. In our case this information is not available, and we had to utilize alternative sources of information. Godt *et al.* (29) have successfully identified cellular targets of the p38 kinase inhibitor SB203580. This inhibitor is structurally related to the NS5A-p58-i (Fig. 1), which also potently inhibits p38 (24). Consequently, we attached compound H479 and A852 to an epoxy-activated Sepharose matrix at the position corresponding to the sulfoxide substituent of SB203580. Whereas 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 position (Fig. 1, compound A852-*m*). We tested the activity of this modified compound (A852-*m*) in an NS5A hyperphosphorylation assay and observed a 2–3-fold reduction of activity after introduction of this modification (data not shown). The affinity matrix was prepared as described under “Experimental Procedures,” and total cell lysate prepared from 10A-IFN cells was incubated with this matrix at high salt concentrations to reduce nonspecific protein binding. The choice of elution conditions enabled us to distinguish between those proteins that bind to the compound with low affinity from those with high affinity. Although 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 for efficient elution.

A Distinct but Overlapping Kinase Profile Is Obtained with Two NS5A Hyperphosphorylation Inhibitors—For the identification of cellular targets of the NS5A-p58-i, cellular lysates prepared from 10A-IFN cells were 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. 2 and Table 1). The elution profiles in the presence of 100 mM ATP with or without 1 mM compound clearly differ 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, non-kinase targets such as tubulin, actin, prohibitin, glutamine synthetase, and aldehyde dehydrogenase were also found to bind the affinity matrix. These proteins bind nucleotides such as ATP or NADH and might associate with the affinity matrix because of their high abundance within the cell. In fact most of these proteins eluted in the presence of ATP alone indicating that binding is because of their affinity for nucleotides rather than for the specific compounds.

The *in vitro* screening of a selected panel of kinases has shown that only four enzymes, CKI- α , CKI- δ , CKI- ϵ , and p38 MAPK, were efficiently inhibited by three NS5A-p58-i (24). In

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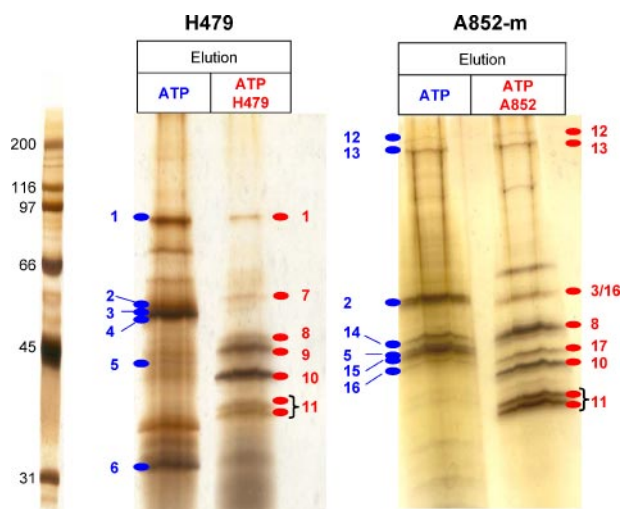


FIGURE 2. Inhibitor affinity chromatography with the NS5A hyperphosphorylation inhibitors. Cellular proteins were passed through a column containing covalently bound H479 (left) or A852-m (right), and proteins were eluted either with ATP or with ATP plus compound. After elution, proteins were concentrated, separated by 10% SDS-PAGE, and visualized by silver staining. Identified proteins are listed in Table 1.

TABLE 1
Identification of proteins isolated by inhibitor affinity chromatography by mass spectrometry analysis

No. of proteins	Protein name	Swiss-Prot accession no.
1	RSK1/2	Q15418/P51812
2	Aldehyde dehydrogenase	P30837
3	JNK2	P45984
4	CaMKII δ	Q13557
5	JNK1	P45983
6	Prohibitin	P35232
7	RIPK2 (receptor-interacting serine/threonine protein kinase 2)	O43353
8	CKI- δ /CKI- ϵ	P48730/P49674
9	Glutamine synthetase	P15104
10	p38- α , - β MAPK	Q16539/Q15759
11	CKI- α	P48729
12	CIT (citron Rho-interacting kinase)	O14578
13	GAK (cyclin G-associated kinase)	O14976
14	GSK3 β (glycogen synthase kinase-3)	P49841
15	Actin	P60709
16	Tubulin	P68366
17	PKA C- α	P17612

this study, we offered a complex mixture of cellular kinases and nucleotide-binding proteins present in crude cell extracts. Despite the high complexity of proteins offered to the affinity matrix, only CKI- α , CKI- δ , CKI- ϵ , and p38 MAPK 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 the H479-matrix, RIPK2 was the only high affinity binding kinase present in the ATP/H479 fraction. Even though we also identified RSK1/2, it seems that this kinase interacts with the H479-matrix with lower affinity because most of the 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 the H479-matrix, we identified, in addition to RSK1/2, also the kinases JNK1, JNK2, and CaMKII δ . For the A852-m-

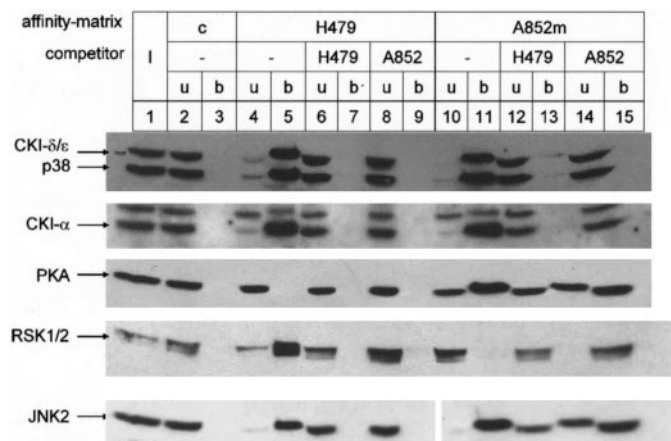


FIGURE 3. Confirmation of binding and different compound affinities of identified kinases by immunoblot analysis. Cellular proteins were bound in batch either to the control matrix (c) or to the affinity-matrix H479 or A852-m as indicated in the *affinity-matrix* lane. The presence of competitive compound 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.

TABLE 2
Calculation of IC₅₀ values of the NS5A hyperphosphorylation inhibitors H479 and A852 for different cellular kinases *in vitro*

	H479 IC ₅₀ (μ M)	A852 IC ₅₀ (μ M)
p38 MAPK	≤ 0.028	≤ 0.024
CKI- δ	1.4	0.4
CKI- α	1.1	1.3
PKA	260	2
JNK1	385	175
JNK2	404	166
RSK2	76	39
CKII	>100	>100

matrix, the high affinity binding kinases present in the ATP/A852 fraction are, in addition to CKI and p38 MAPK, 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, JNK1, and a small amount of PKA in the ATP fraction of the A852-m resin.

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. 3. 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 2). None of the kinases tested bound to the control resin (Fig. 3, 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 (Fig. 3, 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 IC_{50} value of these kinases (see Table 2). Binding and competition of the other kinases reflect the binding affinities already observed in Fig. 2 and can be explained with the IC_{50} value shown in Table 2. PKA binds with high affinity to immobilized A852-m (Fig. 3, lane 11), whereas no binding is detected to the H479 resin (Fig. 3, lane 5). Although A852 efficiently competes in binding ($2000 \times IC_{50}$, Fig. 3, lanes 14 and 15), 2 mM H479 ($7 \times IC_{50}$) is not sufficient to complete competition of PKA binding (lanes 12 and 13). In contrast, RSK binds to the H479 resin (Fig. 3, 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 (Fig. 2). In addition, 2 mM of either A852 or H479 in solution is sufficient to compete for RSK binding to the H479 resin (Fig. 3, lanes 6–9). The IC_{50} 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. 3, 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. 2). The IC_{50} 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. 3, lanes 12 and 13), whereas A852 is able to compete completely (lanes 14 and 15).

Taking all this information together, we have confirmed the binding of some of the kinases by Western blot analysis, and we have shown that affinity of binding shown in Fig. 2 generally correlates with the IC_{50} of the compounds for the kinases.

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. 4. 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 .

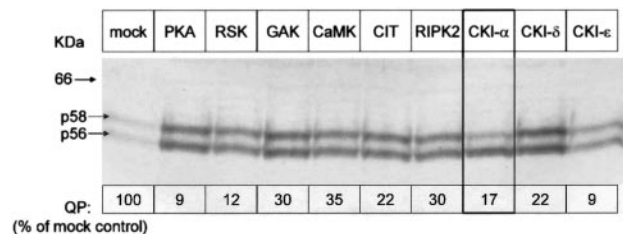


FIGURE 4. CKI- α is the only target kinase important for NS5A hyperphosphorylation. The indicated kinases were silenced in 10A-IFN cells as described under "Experimental Procedures." 48 h after siRNA transfection, 2 μ g of pcD-Bla-wt were transfected, and proteins were expressed using the vaccinia T7 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%). The position of NS5A-p56 and NS5A-p58 and migration of protein standard with 66 kDa, respectively, are indicated on the left.

Hyperphosphorylation of NS5A Occurs upon Incubation with CKI- α in Vitro—The data shown above demonstrate that CKI- α is the only kinase that is a target of both NS5A hyperphosphorylation inhibitors and that 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 *Escherichia coli* as a substrate (Fig. 5). 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* (30, 31). The most interesting result of this experiment is the appearance of a slower migrating band (p58*), which resembles the hyperphosphorylated form of NS5A-p58 produced in cells (Fig. 5, B and C). p58* appeared with time upon incubation with CKI but not with CKII or PKA. Doubling the enzyme concentration of CKII did not result in the production of p58* (data not shown). The reaction is slowed down in the presence of compound H479 with the consequence that the phosphorylation state of NS5A after 4 h of incubation in the presence of compound is comparable with a 20-min incubation without compound. Mutations at serine residue Ser-2204 are frequently found as adaptive mutations and significantly decrease NS5A hyperphosphorylation (20). 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. 5C, lane 4). Interestingly, the same effect could also be observed in the *in vitro* kinase reaction, in which only NS5A is present (Fig. 5C, 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. 5A). 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. 5C, lanes 5–7, hyperphosphorylation of NS5A in

HCV NS5A Is a Direct Substrate of CKI- α

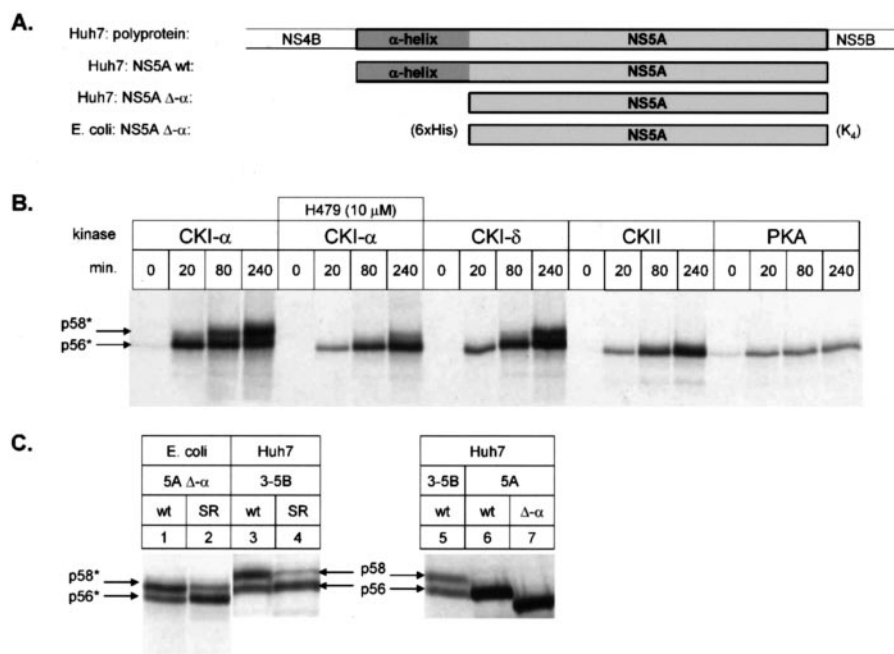


FIGURE 5. NS5A is hyperphosphorylated *in vitro* by casein kinase 1. *A*, schematic presentation of the NS5A sequence used for expression in Huh7 cells or in *E. coli*. Δ - α , N-terminal deletion of amphipathic helix. Purified proteins may contain a histidine tag at the N terminus (6xHis) or a lysine tail at the C terminus (K_4) 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 μ M H479 for 0, 20, 80, or 240 min at room temperature with [γ - 33 P]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. Shown is an autoradiogram. Migration of NS5A-p56, p58, p56*, and p58* is indicated.

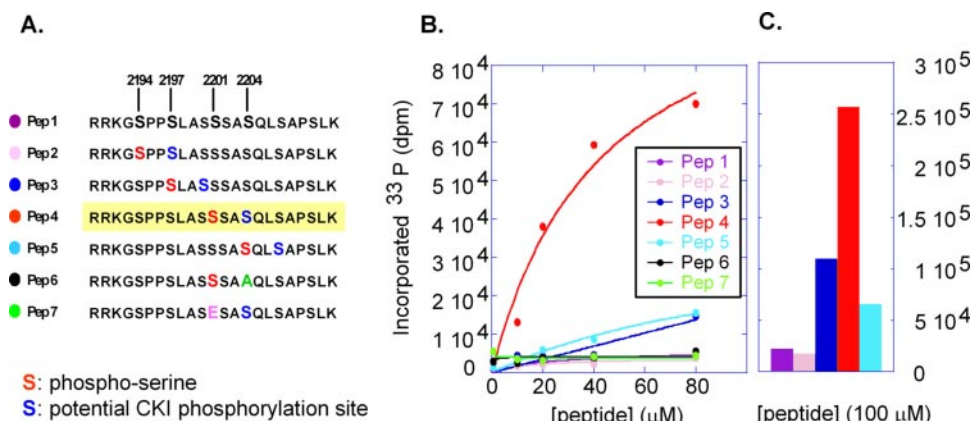


FIGURE 6. Efficient phosphorylation of an NS5A peptide requires pre-phosphorylation of Ser-2201. Seven different peptides spanning the region of the NS5A hyperphosphorylation sites were incubated with CKI as described under "Experimental Procedures." *A*, schematic presentation of the seven peptides. 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. 33 P incorporated in the peptides is shown as disintegrations/min on the y axis at various concentrations of the substrate peptides (x axis).

cells requires the presence of the HCV nonstructural proteins, independent of the N-terminal amphipathic helix.

Ser-2204 Is a Preferred Phosphorylation Site of CKI- α —The experiment shown above clearly demonstrates that NS5A is

directly phosphorylated by CKI- α . Knowing the importance of the serine residues Ser-2197, Ser-2201, and Ser-2204 for NS5A hyperphosphorylation, we investigated whether these serine residues may be phosphorylated by other kinases within the consensus sequence (S/T)(P) X_{1-2} (S/T) (32). Interestingly, the region of hyperphosphorylation is a hot spot for CKI recognition, containing many potential consensus sequences (Fig. 6A). We designed five peptides spanning the region from residue Gly-2193 to Lys-2212, either unphosphorylated (Pep 1) or phosphorylated at a single defined serine residue. These peptides were offered as substrates to CKI in an *in vitro* kinase assay, and the results are shown in Fig. 6B. The activity of different isoforms of CKI on protein substrates depends on the intact tertiary structure of the substrates, whereas the specific activity on peptide substrates is virtually identical (33). We therefore performed the experiment using CKI- δ available from a commercial source. The most efficiently phosphorylated substrate peptide is Pep 4. Phosphorylation of peptide 4 depends on the presence of phosphoserine 2201 (compare peptide 1 with 4). Comparison of the sequence of peptide 4 with the CKI consensus sequence suggests that Ser-2204 is phosphorylated by CKI- δ . This assumption was confirmed by Pep 6. In this peptide Ser-2204 was substituted by alanine, resulting in complete abolition of CKI- δ activity. In Pep 7 we exchanged Ser-2201 with glutamic acid to test whether phosphorylation of Ser-2201 is important or whether the presence of a negative charge is sufficient for phosphorylation of Ser-2204. As shown in Fig. 6B, the S2201E substitution completely abolished the activity of CKI- δ , indicating that phosphorylation of Ser-2201 is required. Similar experiments with recombinant CKII showed no activity on these peptides (data not shown). To confirm that the activity of CKI- α on peptides 1-5 is comparable with the activity of CKI- δ , the experiment was

repeated at a single peptide concentration with the CKI- α isoform. Fig. 6C shows that peptide preferences of the different CKI isoforms are indeed comparable.

DISCUSSION

It is generally accepted that NS5A phosphorylation plays a key role in the regulation of HCV replication. Several kinases have been implicated in NS5A phosphorylation mainly using biochemical experiments (30, 31, 34). We have demonstrated for the first time that CKI- α is directly involved in NS5A hyperphosphorylation in cells (24). CKI- α is a target of the NS5A-specific hyperphosphorylation inhibitors, which were used in an *in vitro* screen of a panel of 60 kinases. In this study, we adopted an alternative methodology, the inhibitor affinity chromatography, to confirm CKI- α as a target of the NS5A-p58-i in a more complex context of other competing cellular kinases and ATP-binding proteins. In addition, other targets not present in the previous screening were identified and evaluated for their effect on NS5A phosphorylation.

Most of the proteins binding to the affinity matrix were kinases, even though non-kinase ligands could be identified. Binding of these non-kinase targets like tubulin, actin, prohibitin and aldehyde dehydrogenase most likely can be explained by their cellular abundance and their general affinity for nucleotides. p38 MAPK and CKI, those kinases that we have shown previously to be inhibited *in vitro* by all three NS5A-p58-i, also bind to both inhibitor-matrices 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 of the different targets varied between the low nanomolar range up to the high micromolar range. The fact that protein kinases that were only moderately inhibited by the compound(s) ($IC_{50} \approx 400 \mu M$) 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, whereas calculation of the IC_{50} value is performed in the presence of 125 μM ATP, intracellular ATP is diluted out during extract preparation, and thus the binding reaction is performed in the absence of competing ATP.

As a matter of fact, some of the identified kinases have already been isolated using the inhibitors SB203580 or pyrido[2,3-*d*]pyrimidine (29, 35). 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 (Fig. 1), and it is therefore not surprising that this set of kinases associates with the NS5A kinase inhibitors as well. All kinases that were identified in the high affinity fractions of the compounds were tested for their effect on NS5A hyperphosphorylation using the method of RNA interference. With the exception of CKI- α , silencing of none of the other kinases changed the NS5A phosphorylation pattern. These results suggest that CKI- α may be the only cellular kinase present in Huh7 cells that is targeted by all NS5A hyperphosphorylation inhibitors and is important for NS5A hyperphosphorylation. It would be possible, however, that the extent of phosphorylation of NS5A by kinase(s) could also be differentially altered in HCV-infected host cells with respect to

that in uninfected host cells. Thus, additional kinases important for NS5A phosphorylation might have been missed with our approach.

Nevertheless, the fact that p38 and probably also RIPK2, CIT, and GAK are efficiently inhibited by these inhibitors might account for undesired off-target activities. PKA is the only kinase tested in this work on which the two compounds A852 and H479 showed a clear difference in potency. At 8 μM concentration, a concentration sufficient to inhibit NS5A hyperphosphorylation and activate replication of wild type Con1 RNA in cells (21), PKA is significantly inhibited by A852 (IC_{50} 2 μM) but not at all by H479 (IC_{50} 270 μM). It was shown previously that A852 activates replication of wild type Con1 HCV RNA less efficiently than H479 (21), 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 IC_{50} values compared with H479 and competes more efficiently in solution (Fig. 4). By comparing the structure of the two compounds, it would be possible that the bulky trifluoromethyl group present in H479 hampers the interaction with the active site of most kinases, thus explaining this observation.

So far, biochemical experiments demonstrated that NS5A is a substrate of several kinases. However, a form of NS5A migrating at a higher molecular weight in an SDS gel, as expected for p58, was never observed (see CKII and PKA in Fig. 5). We noticed here for the first time that *E. coli*-expressed NS5A produces a slower migrating band upon incubation with CKI. 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. 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 obviously does not exclude the possibility that in cells additional kinases might be involved in NS5A phosphorylation or regulation of 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 N terminus of NS5A to the membranes puts constraints 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 constraint, *i.e.* attachment to the membranes via the amphipathic helix, does not facilitate NS5A hyperphosphorylation (Fig. 5C). This might indicate that localization of NS5A at the membranes is indeed important for hyperphosphorylation. Interestingly, CKI- α is found in different membrane compartments, and its activity is differentially regulated by components within these membranes (36).

Correct folding exposes those sites important for basal phosphorylation, which in turn are the basis for hyperphosphorylation. The *in vitro* experiments using the NS5A peptides indicate

that Ser-2204 is a substrate residue for CKI- α . The requirement of pre-phosphorylated Ser-2201 hints to a hierarchical order of phosphorylation. Even though Ser-2201 is not a substrate of CKI- α in the context of a peptide, one cannot exclude the possibility that Ser-2201 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 Ser-2201. In the more natural context of a living cell, this pre-phosphorylation of Ser-2201 might well be performed by a different cellular kinase.

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. This offers for the first time a potent tool with which various phosphorylated forms of NS5A can be dissected and investigated for their different functions in HCV RNA replication and infection.

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Hepatitis C Virus NS5A Is a Direct Substrate of Casein Kinase I- α , a Cellular Kinase Identified by Inhibitor Affinity Chromatography Using Specific NS5A Hyperphosphorylation Inhibitors

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