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Profiling Kinase Activity during Hepatitis C Virus Replication Using a Wortmannin Probe

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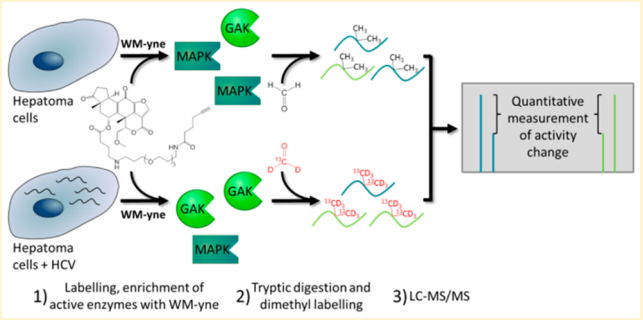
ABSTRACT: To complete its life cycle, the hepatitis C virus (HCV) induces changes to numerous aspects of its host cell. As kinases act as regulators of many pathways utilized by HCV, they are likely enzyme targets for virally induced inhibition or activation. Herein, we used activity-based protein profiling (ABPP), which allows for the identification of active enzymes in complex protein samples and the quantification of their activity, to identify kinases that displayed differential activity in HCV-expressing cells. We utilized an ABPP probe, wortmannin-yne, based on the kinase inhibitor wortmannin, which contains a pendant alkyne group for bioconjugation using bioorthogonal chemistry. We observed changes in the activity of kinases involved in the mitogen-activated protein kinase pathway, apoptosis pathways, and cell cycle control. These results establish changes to the active kinome, as reported by wortmannin-yne, in the proteome of human hepatoma cells actively replicating HCV. The observed changes include kinase activity that affect viral entry, replication, assembly, and secretion, implying that HCV is regulating the pathways that it uses for its life cycle through modulation of the active kinome.



KEYWORDS: *activity-based protein profiling, kinase probes, wortmannin, hepatitis C, MAPK pathway, host−virus interactions*

The hepatitis C virus (HCV) is a small, positive-sense RNA virus consisting of a 3′ and 5′ untranslated region (UTR) flanking a coding region for a polyprotein, later cleaved into three structural and seven nonstructural proteins.[1] It currently infects between 2 and 3% of the global population and presents a global health threat in both developed and undeveloped countries.[1] No vaccine is currently available.[2] Although second-wave direct-acting antivirals (DAAs) show promise, their therapeutic potential is limited by the virus's high heterogeneity, which leads to a low barrier to drug resistance and the development of escape mutants. Furthermore, DAAs display different degrees of success in producing a significant reduction in viremia across the various HCV genotypes.[2] The development of host-targeting antivirals addresses this issue, targeting aspects of the host cell required for viral propagation and thereby reducing the possibility of escape mutants when used in conjunction with DAAs. To propagate efficiently, HCV must alter the host cell machinery it uses to enter, replicate, assemble, and secrete from the cell.[1,3] Investigating host factors required for replication and infection allows for a better understanding of how HCV and related RNA viruses utilize host cells and can serve as targets for the development of host-targeting antivirals. Many of the biochemical pathways HCV needs to manipulate to create a pro-viral environment are controlled by kinases, which are therefore attractive targets both for the virus and for drug therapies.

HCV has been shown to depend on the activity of a wide variety of kinases. These kinases include C-Src kinase (CSK),[4] cell cycle regulatory kinases,[5] choline kinases,[6] and phosphatidylinositol 4-kinases.[7−13] Kinases involved in the AKT-PI3K pathway,[14,15] mitogen-activated protein kinase pathway,[6,14] and apoptotic pathway,[14] as well as kinases that act as growth factors and initiation factors,[6] have also been implicated. Alternatively, certain kinases have been shown to act as HCV suppressors, such as protein kinase C and casein substrate in neurons 1 (PACSIN 1), cyclin-dependent kinase regulatory subunit, and a kinase in the MAPK pathway, mitogen-activated protein kinase kinase 5.[16]

It has been shown that, as a result of this dependence, HCV modifies the activity of numerous kinases to promote the production of new virions. The activities of pro-viral kinases, such as phosphoinositide kinases PI3K,[17−19] PI4KIIIα, and, potentially, PI4KIIIβ,[12,20−23] are up-regulated by HCV to create an environment more favorable for the various stages of the viral life cycle. To the same end, HCV is also known to suppress kinase activity to inhibit the NF-κB pathway, the insulin signaling pathway, and immune response, where affected proteins include protein kinase R and inducible I κB

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kinase.[6,24−26] However, the effect of viral infection on the activity of many kinases shown to play a role in HCV infection has yet to be determined. The identification of kinases that display differential activity in response to HCV infection allows us to elucidate the mechanism by which HCV propagates and to identify novel drug targets not previously associated with HCV.

As the activities of kinases are regulated by a number of post-translational regulatory mechanisms, it is necessary to use methods that go beyond traditional abundance-based proteomics to obtain an accurate portrait of changes in the state of the cell. Activity-based protein profiling (ABPP) uses probes with active-site directed reactive groups, typically based on previously identified inhibitors, to assess the catalytic ability of target enzymes.[27,28] The development of rapid, bioorthogonal "click" chemistry has made it possible to attach the bulky reporter tag to the probe after labeling to increase the permeability of the activity-based probe. ABPP can be combined with stable isotope dimethyl labeling followed by LC-MS/MS, a high-throughput method by which proteins can be identified and their relative quantities across two samples compared, to identify enzymes that display differential activity between two samples.[29,30] ABPP has previously been used in the labeling and identification of kinases[23,31−34] and in the context of viral infections[23,35−40] to label and quantify the activity of enzymes and kinases specifically.[23,36]

Wortmannin (Figure 1A) is a small fungal-derived molecule that acts as an irreversible inhibitor against a range of kinases in

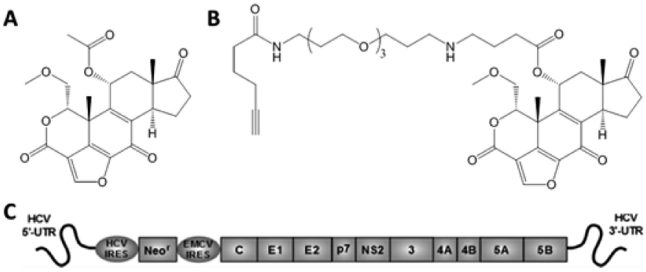

Figure 1. Chemical structures of probes and protocol used to target active kinases: (A) wortmannin; (B) wortmannin-derived clickable ABPP probe, wortmannin-yne, containing a bio-orthogonal alkyne moiety; (C) schematic representation of the HCV model, bicistronic full-genomic replicon with an S2204I adaptive mutation in NS5A (Huh7.5-FGR).

the phosphoinositide kinase family of enzymes, as well as members of the protein-phosphorylating phosphoinositide 3-kinase related kinase (PIKK) family.[41] It has also been shown to have antiviral properties against HCV[7,12] and should therefore be capable of targeting kinases relevant to the life cycle of HCV. Wortmannin inactivates its target enzymes through the formation of a covalent bond with a catalytic lysine residue.[42] The formation of this covalent bond between wortmannin and an active enzyme allows a modified wortmannin molecule to act as a selective label of specific active kinases. It has previously been modified to act as an activity-based probe and has been shown to report on the activity PI3K, polo-like kinase of 1, and DNA-dependent protein kinases.[43,44] In this paper, we used a wortmannin-based probe, wortmannin-yne (Figure 1B), to investigate the activity of kinases in response to HCV infection. The activity-dependent labeling of a range of kinases is demonstrated, and we show that HCV induces a change in the activity of several kinases implicated in viral infection, metabolism, hepatic disorders, and cell stress response.

RESULTS AND DISCUSSION

To understand the dependence of HCV on host factors and to develop new monitoring and treatment strategies, it is necessary to identify novel host−virus interactions. To this end, we have searched for host factors that are required for viral propagation. The perturbation of individual enzymes or pathways provides useful clues as to the identity of targetable host factors that are required by the virus. By measuring catalytic activity, the functional end-point of all enzymes, and also the effector of the methods by which enzymatic activity can be regulated, ABPP methods provide a more accurate portrait of the proteome alterations made by HCV.

Proteome Labeling Using Wortmannin-yne. To test the efficacy of wortmannin-yne as a probe in a complex mixture of proteins, Huh7.5 cell lysates were labeled in vitro with various concentrations of wortmannin-yne and tagged with a fluorescent marker. Wortmannin-yne labeled numerous targets, and showed most effective labeling at 10 μM. Competitive inhibition with wortmannin and heat denaturation both resulted in a decrease in wortmannin-yne labeling (Figure 2).

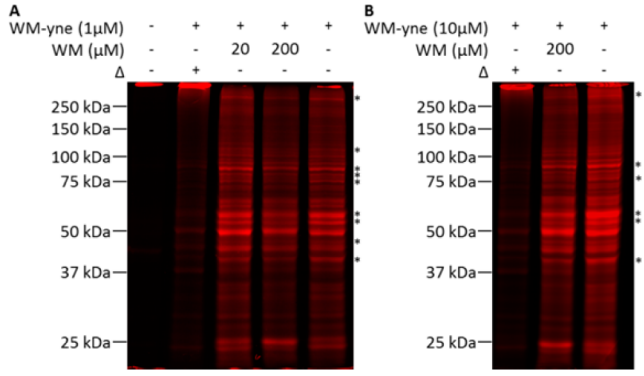

Figure 2. Wortmannin-yne labeling of Huh7.5 proteome. Huh7.5 cell lysates were pre-incubated with the competitive inhibitor wortmannin (WM) at 37 °C for 30 min or heat denatured for 10 min (symbolized by the Δ) before incubation with increasing concentrations of wortmannin-yne (WM-yne) and subsequent attachment of a fluorescent rhodamine tag via click chemistry. Labeled proteomes were visualized by fluorescent scanning at 65% sensitivity for samples labeled with 1 μM wortmannin-yne and at 50% sensitivity for samples labeled by 10 μM wortmannin-yne. Proteins exhibiting decreased labeling in the presence of inhibitor are marked by an asterisk.

The inhibition of wortmannin-yne labeling by protein denaturation indicates that wortmannin-yne acts as an activity-based probe, whereas the inhibition of numerous bands by the competitive inhibitor wortmannin indicates that the target range of the probe retains significant similarity to the parent molecule. The presence of uninhibitable bands is due to structural differences between the inhibitor wortmannin and the probe-wortmannin-yne, which results in a slight difference in the range of enzymes targeted, consistent with previous results.[43,44] The Coomassie stain showed similar band intensities in every lane, indicating identical amounts and composition of protein in each sample (not shown).

Wortmannin-yne Target Identification by Comparative ABPP. To assess changes in kinase activity caused by

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Table 1. Kinases Displayed Down-Regulated Activities in Hepatoma Cells Expressing the HCV Full-Genomic Replicon

protein name	gene name	accession no.	spectral counts[a]	dimethyl labeling		
				trial 1[b]	trial 2[b]	mean
casein kinase II subunit α	CSNK2A1	Q5U5J2	6/12	0.4	0.8	0.6
bifunctional ATP-dependent dihydroxyacetone kinase	DAK	Q3LXA3	2/1	0.5	0.7	0.6
cyclin-dependent kinase 18	CDK18	Q07002		0.6	0.8	0.7
phosphatidylinositol 5-phosphate 4-kinase type-2 α	PIP4K2A	P48426		0.4	0.9	0.7
casein kinase II subunit α	CSNK2A2	P19784	3/5	0.6	0.8	0.7
cyclin-dependent kinase 1	CDK1	P06493		0.5	0.8	0.7
UMP-CMP kinase	CMPK1	P30085	4/6	0.7	0.7	0.7
mitogen-activated protein kinase 1	MAPK1	P28482	3/7	0.8	1.0	0.9
dual specificity mitogen-activated protein kinase kinase 1	MAP2K1	Q02750	0/1			
RAC-β serine/threonine-protein kinase	AKT2	P31751	0/1			
serine/threonine-protein kinase PAK 2	PAK2	Q13177	0/1			
cyclin-dependent kinase 2	CDK2	P24941	0/1			

[a]Kinase activity is reported as the fraction of the spectral count from Huh7.5-FGR cells over the spectral count of Huh7.5 cells. [b]The reported ratio for one trial is the mean of a reciprocal pair of samples. One sample contained "light"-labeled peptides from Huh7.5 cells and "heavy"-labeled peptides from Huh7.5-FGR cells, and the other contained "heavy"-labeled peptides from Huh7.5 cells and "light"-labeled peptides from Huh7.5-FGR cells.

Table 2. Kinases That Displayed Up-Regulated Activities in Hepatoma Cells Expressing the HCV Full-Genomic Replicon

protein name	gene name	accession no.	spectral counts[a]	dimethyl labeling		
				trial 1[b]	trial 2[b]	mean
nucleoside diphosphate kinase A	NME1	P15531	1/0	2.2	2.8	2.5
cyclin-G-associated kinase	GAK	O14976		1.7	2.4	2.1
phosphoenolpyruvate carboxykinase [GTP], mitochondrial	PCK2	Q16822	1/1	1.3	2.6	2.0
calcium/calmodulin-dependent protein kinase type II subunit δ	CAMK2D	Q13557	2/2	1.7	1.9	1.8
6-phosphofructokinase, liver type	PFKL	P17858		1.6	1.7	1.7
6-phosphofructokinase, muscle type	PFKM	P08237	3/1	1.0	1.2	1.1
RAC-β serine/threonine-protein kinase	AKT2	P31751	1/0			
calcium/calmodulin-dependent protein kinase type 1	CAMK1	Q14012	1/0			
calmodulin	CALM1	H0Y7A7	1/0			
cAMP-dependent protein kinase type I-α regulatory subunit	PRKAR1A	P10644	1/0			
serine-protein kinase ATM	ATM	Q13315	1/0			
cell cycle progression restoration protein 2	TBRG4	Q969Z0	1/0			
glycogen synthase kinase-3 β	GSK3B	P49841	2/0			

[a]Kinase activity is reported as the fraction of the spectral count from Huh7.5-FGR cells over the spectral count of Huh7.5 cells. [b]The reported ratio for one trial is the mean of a reciprocal pair of samples. One sample contained "light"-labeled peptides from Huh7.5 cells and "heavy"-labeled peptides from Huh7.5-FGR cells, and the other contained "heavy"-labeled peptides from Huh7.5 cells and "light"-labeled peptides from Huh7.5-FGR cells.

HCV, naïve Huh7.5 and Huh7.5 cells expressing the full genomic replicon, Huh7.5-FGR (Figure 1C), were lysed and the proteomes labeled in vitro with wortmannin-yne. The lysates were subsequently tagged with biotin-azide via copper-catalyzed click chemistry. The labeled proteome was separated from the unlabeled proteome by affinity pull-down and analyzed by LC-MS/MS. The relative kinase activities of the HCV replicon-containing and naïve samples were determined by calculating the ratio of the spectral counts from the two samples. Due to the low number of spectral counts per hit, this method yielded only semiquantitative results (Tables 1 and 2).[45]

To confirm our results, we used a method that combines ABPP with dimethyl labeling followed by mass spectrometry.[30] As shown in Figure 3, the tryptic digests of the wortmannin-yne labeled proteomes were dimethylated with either "heavy" or "light" formaldehyde, then combined and analyzed by LC-MS/MS. To eliminate bias caused by the nature of the stable isotope label, the dimethyl labeling was performed in reciprocal pairs: one sample contained "light"-labeled peptides from Huh7.5 cells and "heavy"-labeled peptides from Huh7.5-FGR cells, and the other pair contained "heavy"-labeled peptides from Huh7.5 cells and "light"-labeled peptides from Huh7.5-FGR cells. The peak intensities for the identified proteins were used to calculate the ratio of the enzymes' activities between Huh7.5 and HCV-containing cells. Wortmannin-yne labeling demonstrated differential activity in a large number of enzymes, enabling the assessment of HCV-induced changes in activity of numerous enzymes using a single probe.

TOPPGENE enrichment analysis was conducted on protein hits. Analysis of kinases displaying differential activity showed that HCV activates kinases involved in the mitogen-activated protein kinase (MAPK) pathway, cell cycle regulation, and tumor suppression (Table 3). Other individual kinases that had previously been associated with HCV entry, replication, and assembly were also identified and demonstrated differential activity in the presence of HCV (Tables 1 and 2).

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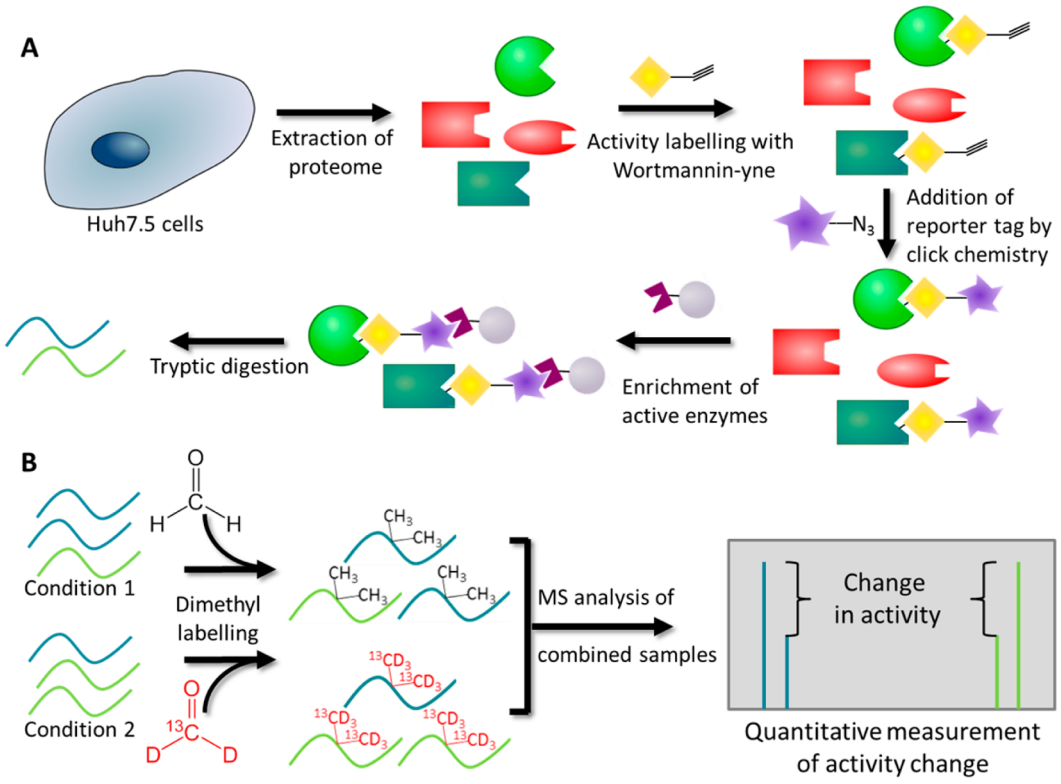

Figure 3. Scheme of the activity-based profiling methods used to identify differentially active kinases. (A) The active proteome isolated from naïve hepatoma cells or hepatoma cells stably expressing an HCV replicon is labeled by wortmannin-yne and subsequently attached to an affinity tag. The tagged proteome was isolated by affinity pull-down digested by trypsin. Peptides could be analyzed by LC-MS/MS or undergo further manipulation. (B) Additional steps were used in ABPP-dimethyl labeling. Digested peptides are labeled with either "light" or "heavy" formaldehyde. The samples are mixed and the targets identified and quantified by LC-MS/MS.

Table 3. Pathways Identified by TOPPGENE Gene Enrichment Analysis

pathway name	*p* value	genes from input	genes in annotation	ratio of labeled to known pathway genes
ERK activation	7.81×10^{-8}	3	5	0.60
RAF/MAP kinase cascade	9.31×10^{-7}	3	10	0.30
condensation of prometaphase chromosomes	1.70×10^{-6}	3	12	0.25
RB tumor suppressor/checkpoint signaling in response to DNA damage	2.21×10^{-6}	3	13	0.23
Ca^{2+}/calmodulin-dependent protein kinase activation	2.81×10^{-6}	3	14	0.21
GRB2 events in EGFR signaling	2.81×10^{-6}	3	14	0.21
SOS-mediated signaling	2.81×10^{-6}	3	14	0.21
signaling to p38 via RIT and RIN	3.51×10^{-6}	3	15	0.20
SHC-mediated signaling	3.51×10^{-6}	3	15	0.20
SHC1 events in EGFR signaling	3.51×10^{-6}	3	15	0.20
cell cycle: G1/S check point	2.79×10^{-7}	4	28	0.14
signal transduction by L1	7.07×10^{-7}	4	35	0.11
NFAT and hypertrophy of the heart (transcription in the broken heart)	9.38×10^{-10}	6	54	0.11
fMLP induced chemokine gene expression in HMC-1 cells	8.90×10^{-7}	4	37	0.11
Fc ε receptor I signaling in mast cells	1.11×10^{-6}	4	39	0.10
IFN-γ pathway	1.23×10^{-6}	4	40	0.10
bioactive peptide induced signaling pathway	1.65×10^{-6}	4	43	0.09
IL-7 signaling pathway	1.81×10^{-6}	4	44	0.09
glioma	1.83×10^{-7}	5	65	0.08
ErbB signaling pathway	1.88×10^{-8}	6	88	0.07

Kinases Involved in Insulin Signaling. The protein kinase Akt acts as a central hub between extracellular signals and the regulation of several cellular pathways, the most significant of which is insulin signaling.[46] Comparative ABPP by MS shows an HCV-mediated decrease in the activity of protein kinase Akt activity (Table 1). Similar results were seen after western immunoblotting on activity-enriched proteomes. Glycogen synthase kinase 3 beta (GSK3β), a downstream

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target of insulin signaling that inhibits insulin-mediated glycogen synthesis,[47] displayed increased activity in HCV-expressing cells, (Table 2). These results are consistent with hypothesized mechanisms for hepatitis C associated insulin resistance[47,48] and agree with previous research, which showed that HCV proteins decrease Akt activity while increasing GSK3β activity.[17,49−54] Contrary to these findings, other researchers have shown that HCV increases the activity of Akt[14,55] and that GSK3β is inactivated in cells expressing either the HCV core protein[56] or the subgenomic HCV replicon.[57]

Prior studies assessed the activities of Akt and GSK3β by measuring the activating phosphorylations of Akt Thr308 and Ser473 and the inactivating phosphorylation of GSK Ser9 by Akt. However, recent research has shown that this is not an accurate measurement of the activity of these kinases, as additional post-translational modifications can modulate Akt or GSK3β independently of phosphorylation.[46,47,58,59] The use of comparative ABPP avoids these potential sources of inaccuracy, as the activities of the enzymes themselves are necessary for labeling.

Kinases Essential to Host Cell Metabolism. Key metabolic regulatory kinases displayed differential activity in the presence of HCV. Phosphoenolpyruvate carboxykinase (PCK2), a key rate-limiting enzyme in hepatic gluconeogenesis,[55] showed increased activity in HCV-infected cells. This agrees with previous research, which showed that PCK2 has higher transcription and protein levels in HCV-expressing cells.[55,60] Additionally, two isoforms of ATP-dependent 6-phosphofructokinase (PFKM and PFKL), an enzyme whose activity positively regulates the rate of glycolysis,[61] were also labeled by the wortmannin probe. Quantification by spectral counting showed a three-fold increase in PFKM activity in HCV-replicon cells. This result was not supported by the intensity comparison method used in the dimethyl labeling, which showed only a modest increase in activity. The dimethyl-labeling experiments did, however, show an average 66% increase in the activity of PFKL, the more abundantly expressed liver isoform. Increases in phosphofructokinase activity have previously been shown in biopsy samples from patients suffering from acute hepatitis.[62]

Kinases Involved in the MAPK Pathway. Both spectral counting and dimethyl-labeling quantification revealed altered activity in MAPK pathways kinases. As shown in Table 1, the activities of mitogen-activated protein kinase 1 (MAPK1) and dual specificity mitogen-activated protein kinase kinase 1 (MAP2K1), the kinase that activates MAPK1,[63] were down-regulated in HCV-containing cells compared to naïve cells (Table 1). Pathway enrichment analysis of differentially active kinases indicates that the MAPK pathway is strongly inhibited by HCV (Table 3).

To confirm that MAPK1 activity is decreased in HCV-replicon samples, active kinases of Huh7.5 and Huh7.5-FGR cells labeled with wortmannin-yne were isolated by affinity enrichment and detected by western immunoblotting to confirm both the wortmannin-yne labeling of MAPK pathway kinases and the dimethyl-labeling-based quantification of activity. MAPK1 showed a decrease in activity in Huh7.5-FGR cells, which agrees with the mass spectrometry quantification (Figure 4). A decrease in MAPK1 abundance was also observed, suggesting that the decrease in MAPK1 activity is due to lower transcription levels and not solely due to post-translational regulation.

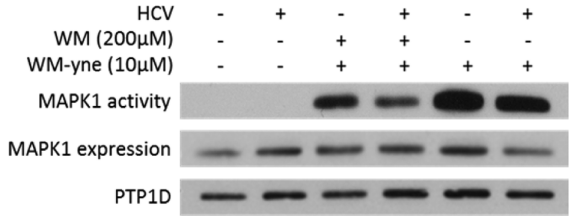

Figure 4. Western blot analysis of wortmannin-yne pulldown in HCV-expressing cell lines. Cell lysate proteins probed with wortmannin-yne were affinity enriched, separated using SDS-PAGE, and probed with antibodies against MAPK1 and PTP1D.

These results are in agreement with previous studies, which have shown that expression of the HCV replicon inhibits the activation of the MAPK pathway via interaction between nonstructural protein 5A (NS5A) and upstream regulators and that the chemical inhibition of MAP2K1 increases HCV replication.[14,63−66] In addition to confirming that the MAPK pathway is inhibited by HCV replication, these results show that the wortmannin-yne probe is a valuable tool, which can be used to assess the activation of the MAPK pathway in Huh7.5 cells.[67,68]

Kinases Implicated in Cell Cycle Regulation. HCV has previously been shown to decrease the proliferation of hepatocytes by inhibiting cell cycle progression.[69−72] Comparative ABPP with wortmannin-yne revealed multiple perturbations in the activity of cell cycle regulatory proteins. Cyclin-dependent kinase 1 (CDK1), which facilitates the onset of mitosis,[73] and cyclin-dependent kinase 2 (CDK2), which promotes passage through the G1/S barrier,[74] both displayed decreased activity in the presence of HCV (Table 1). This is in agreement with previous research, which showed that chemical inhibition of CDK2 increases viral replication.[64] Cell cycle restoration protein 2 (TBRG4), a kinase that has been shown to block cell cycle arrest at the G1/S barrier,[75] was activated in the presence of HCV (Table 2). Altogether, this suggests that in the presence of HCV, normal cell cycle progression is perturbed via the inhibition of CDK1 and CDK2 and that cell cycle progression is rescued by the activation of the cell cycle progression restoration protein 2.

Kinases regulating apoptosis also displayed differential activation. Serine protein kinase ATM (ATM) demonstrated higher activity in HCV-expressing cells (Table 2). ATM is a regulator of multiple pathways leading to cell cycle arrest and apoptosis. It has previously been shown to be activated and promote cell cycle arrest and apoptosis in response to high lipid levels and oxidative stress, both conditions caused by HCV.[1,76,77] The activities of two subunits of casein kinase II, (CSNK2) another kinase that has been shown to play a role in cell cycle arrest and promotion of apoptosis,[78] were decreased in the presence of HCV (Table 1). Our data suggest that the high lipid levels and oxidative stress caused by HCV activate ATM, promoting cell cycle arrest and apoptosis. The activation of the pro-apoptotic pathways may be countered in part by the inhibition of CSNK2, as the depletion of CSNK2 has previously been shown to bypass cell cycle arrest.[78]

Kinases Associated with the Assembly of HCV Infectious Particles. Two kinases identified by wortmannin-yne labeling, CSNK2 and cyclin-G associated kinase (GAK), have previously been associated with HCV assembly. GAK is a kinase that has been shown to promote both viral entry and viral assembly, but does not affect replication.[79,80] Table 2

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shows that it displayed increased activity in Huh7.5-FGR cells, suggesting that GAK activity is up-regulated by HCV to promote efficient assembly of viral particles and to help enable viral entry during the cycle of infection.

Assembly of HCV infectious particles is regulated by the viral protein NS5A, which acts as a switch between HCV replication and assembly, promoting virion assembly when hyperphosphorylated.[81] CNSK2 has been shown to phosphorylate NS5A and promote the switch from viral replication to viral assembly.[81] Our results indicate that CSNK2 displayed reduced activity in Huh7.5-FGR cells (Table 1), which replicate the HCV genome but do not assemble or secrete infectious particles.[82] Previous research has shown that a decrease in casein kinase activity can increase the production of infectious viral particles.[83] Taken together, this suggests that in cells in which HCV replication occurs, CSNK2 activity is down-regulated to promote the continued replication of the virus. This is consistent with the notion that CNSK2 acts as a master regulator of replication of HCV.

In this paper, we have shown that wortmannin-yne is capable of acting as a probe for high-throughput identification of individual kinases that have altered enzyme activity during HCV replication. As numerous kinases can be labeled by wortmannin-yne, this probe is a valuable tool that can be used to assess differences in both the activity of individual kinases and activation of signaling pathways, for instance, the insulin signaling pathway and the MAPK pathway. Using wortmannin-yne to perform activity-based profiling of HCV genomic replicon expressing cells, we have shown that HCV replication inhibits MAPK signaling and misregulates other enzymes, such as kinases involved in cell cycle control and in tumor suppression, as summarized in Figure 5. These kinases

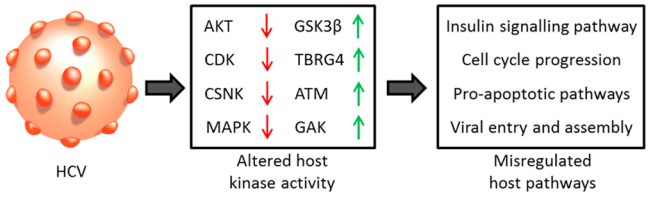

Figure 5. HCV-induced change in kinase activity affects host and host−virus interactions. RAC-β serine/threonine-protein kinase (AKT), glycogen synthase kinase 3-β (GSK3β), cyclin-dependent kinases (CDK), cyclin-G associated kinase (GAK), cell cycle progression restoration protein (TBRG4), casein kinase (CSNK), serine protein kinase ATM (ATM), and mitogen-activated pathway kinases (MAPK) displayed altered activity in HCV-replicon containing cells when probed with activity-based probe wortmannin-yne, leading to the misregulation of host pathways.

represent not only biomarkers for HCV infection but also potential targets for the development of new drug therapies. The misregulation of kinase signaling appears to be important both for the remodeling of the cell by HCV and for the host cell's response to these alterations. These changes occur in the host cell as a result of the expression of the complete viral proteome and of viral replication and, as such, can be assumed to be representative of the effects of HCV infection. The replicon system used herein expresses all of the HCV genome and forms active replication complexes; however, it does not assemble or secrete virions. Thus, there may be some additional changes within the active kinome during a real infection in the human liver in the context of the changes in enzyme activity measured herein. We are currently exploring the mechanistic aspects of these changes in activity.

METHODS

Wortmannin-yne was synthesized as previously reported.[23] The inclusion of the tethered alkyne was shown to not adversely affect targeting of wortmannin. Wortmannin-yne continues to covalently bind to the active sites of phosphatidylinositide and other kinases.[23]

Cell Culture and Reagents. The human hepatoma cell line Huh-7.5 was grown in DMEM supplemented with 100 nM nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% FBS (CANSERA, Rexdale, ON, Canada). The Huh-7.5 cells stably expressing the full genomic replicons (genotype 1B, con1), a kind gift from Dr. Charles Rice (Rockefeller University, New York, NY, USA), were maintained in the same culture medium supplemented with 250 μg/mL G418 Geneticin (GIBCO-BRL, Burlington, ON, Canada).

Proteome Extraction and Labeling. Confluent cells (90−100%) were washed and pooled in ice-cold cell culture lysis buffer (1% Triton-X, 10 mM sodium phosphate buffer (PBS), pH 7.4). Cells were then lysed by sonication (30% duty cycle, 15 pulses, Sonifier 250, Branson Ultrasonic). The proteome extract was cleared by ultracentrifugation at 20000g and 4 °C for 5 min, quantified with the DC protein assay (Bio-Rad), and diluted to 1 mg/mL for the fluorescent gels or to 2 mg/mL for the activity pulldowns. Proteome samples were treated with 10 μM wortmannin-yne for 1 h at room temperature. Competitive inhibition samples were incubated with 200 μM wortmannin for 30 min at 37 °C prior to labeling. Copper-catalyzed azide−alkyne click chemistry and streptavidin enrichment of labeled samples were performed as previously described,[84] with the addition of an acetone precipitation step after the click reaction.

Preparation for Mass Spectrometry. On-bead samples were washed with 50 mM ammonium bicarbonate (ABC) and reduced with 10 mM DTT in 50 mM ABC for 15 min at 65 °C. Samples were rotated with 25 mM iodoacetamide in the dark at room temperature for 30 min. The samples were pelleted, the supernatant was discarded, and the beads were washed with ABC. Samples were digested for 16 h in 10 ng/μL trypsin in ABC and analyzed by mass spectrometric analysis.

Dimethyl Labeling. "Light"-labeled peptides were mixed with formaldehyde (0.16%) and sodium cyanoborohydride (0.025 M). "Heavy"-labeled peptides were mixed with ^{13}C-deuterated formaldehyde (0.16%) and sodium cyanoborodeuteride (0.025M). The samples were rotated for 1 h at room temperature, and the reaction was quenched by the sequential addition of ammonia solution (0.13%) and formic acid (0.32%). Samples were mixed and cleaned in a reverse-phase tC18 Sep-Pak column (Waters, Milford, MA, USA).

Reversed-Phase Liquid Chromatography (RP-LC). An Agilent 1100 capillary-HPLC system (Agilent Technologies, Santa Clara, CA, USA) was hooked up with an LTQ-Orbitrap mass spectrometer (Thermo Electron, Waltham, MA, USA). The solvent system consists of buffer A (0.1% formic acid (FA) in water) and buffer B (0.1% FA in acetonitrile). Dried-down protein digest was acidified with 0.5% (v/v) FA and loaded on a 75 μm i.d. × 100 mm fused silica analytical column packed in-house with 3 μm ReproSil-Pur C18 beads (100 Å; Dr. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 2 μL/min for 15 min. A flow of 20 μL/min from HPLC was split into 200

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nL/min to perform the peptide separation. Gradient elution was set as 5−35% buffer B over 2 h for the on-bead digestion and over 1 h for the in-solution digestion, followed by 2 min at 100% buffer B and 10 min at 2% buffer B to re-equilibrate for the next run.

MS Analysis. An LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA), equipped with a nanoelectrospray interface, was operated in positive ion mode. The spray voltage was set to 2.0 kV and the temperature of the heated capillary was 200 °C. The instrument method consisted of one full MS scan from m/z 300 to 1700, followed by data-dependent MS/MS scan of the five most intense ions, a dynamic exclusion repeat count of 1 in 30 s, and a exclusion duration of 90 s. The full mass was scanned in Orbitrap analyzer with R = 60000 (defined at m/z 400), and the subsequent MS/MS analyses were performed in the LTQ analyzer. All of the measurements in the Orbitrap mass analyzer were performed with a real-time internal calibration by the lock mass of background ion 445.120025 in order to improve mass accuracy. The charge state rejection function was enabled, and charge states with unknown and single charge state were excluded for subsequent MS/MS analysis. All data were recorded with Xcalibur software (ThermoFisher Scientific, San Jose, CA, USA).

Data Analysis, Mascot. All raw files were converted into.mgf files by Proteomics Tools7 and searched using Mascot. Cysteine carbamidomethylation was selected as a fixed modification and the methionine oxidation and protein N-terminal acetylation were selected as variable modifications. Enzyme specificity was set to trypsin, allowing for up to two missing trypsin cleavages not allowing for cleavage of N-terminal to proline. The precursor ion mass tolerances were 7 ppm, and fragment ion mass tolerance was 0.8 Da. The .dat files generated by Mascot were parsed and filtered by BuildSummary4 using a peptide FDR of 1% and a minimum length of six amino acids for peptide identification. All of the identifications from reversed database were removed.

Western Blotting. Labeled proteins were removed from beads by boiling at 95 °C in gel loading buffer (0.1 M Tris, pH 6.8, 10% glycerol, 4% SDS, 0.02% bromophenol blue, 30 mM DTT) for 10 min. Samples were resolved by SDS-PAGE under reducing conditions (10% gel) and transferred to a Hybond-P PVDF membrane (Amersham Biosciences). The membranes were blocked using 2.5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) with 0.05% Tween-20. Membranes were incubated with MAPK1 (1:500) (Santa Cruz), Akt (1:500) (Cell Signaling), or PTP1D (1:4000) (BD Biosciences) in 3% milk in TBST at 4 °C. Membranes were subsequently incubated for 1 h with donkey or mouse monoclonal antibodies (Jackson ImmunoResearch Laboratories) diluted in 3% milk in TBST. Signal was generated using the ECL Plus Western Blotting System (GE Healthcare) as recommended by the manufacturer.

In Vitro 1D Fluorescent Gel Analysis. Cell lysates were treated with 10 μM wortmannin-yne in DMSO for 1 h at room temperature. The copper-catalyzed azide−alkyne click reaction was performed by incubation with click buffer (200 μM rhodamine azide, 2 mM TCEP-HCl, 200 μM TBTA, 2 mM $CuSO_4$) at 25 °C for 1 h with mixing. Following the incubation, the reaction was quenched by acetone precipitation, and the sample was dried and resolved by SDS-PAGE using a 10% acrylamide gel. Gels were scanned with FM BIOIII Mutiview scanner (Hitachi) and stained with Coomassie.

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Notes

The authors declare no competing financial interest.

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