

Hepatitis C Virus Nonstructural Proteins Inhibit Apolipoprotein B100 Secretion

Angela M. Domitrovich², Daniel J. Felmlee^{1,2}, and Aleem Siddiqui^{1,2}

¹Moores UCSD Cancer Center, University of California at San Diego, 3855 Health Sciences Drive #0803, La Jolla, CA 92093; and ²Program in Molecular Biology, Department of Microbiology, mail stop 8333, University of Colorado Health Sciences Center at Fitzsimons, P.O. Box 6511, Aurora, CO 80045

Running title: HCV inhibits apoB100 secretion

Address correspondence to: Aleem Siddiqui, Tel. 858-822-1750; Fax: 858-822-1747; E-Mail: asiddiqui@ucsd.edu

Host genes involved in lipid metabolism are differentially regulated during the early stages of hepatitis C virus (HCV) infection. The majority of lipids synthesized in the liver are exported to other tissues in the form of lipoproteins. The formation of these lipoproteins is dependent upon the association of triglycerides with apolipoprotein B100. Using the HCV subgenomic replicon expression system, we show that secretion of apoB100 is significantly reduced. Inhibition of apoB100 degradation by ALLN did not improve secretion. Triglyceride levels, as well as microsomal triglyceride transfer protein (MTP) mRNA and activity levels were reduced in replicon-expressing cells indicating potential reasons for the observed decrease. Further evidence is presented for the interaction between the HCV nonstructural protein 5A (NS5A) and apoB100. These results provide further insight into the alteration of lipid metabolism by HCV.

Hepatitis C virus (HCV) infection is a major health problem worldwide. HCV infection causes chronic hepatitis in up to 60-80% of infected adults (1) and is associated with steatosis, cirrhosis and hepatocellular carcinoma (2). The HCV viral genome consists of a positive sense single-stranded RNA, 9.6 kb in length, and encodes a polyprotein precursor of approximately 3000 amino acids (3). The resulting polyprotein is cleaved into at least three structural proteins (core, E1 and E2) and a variety of nonstructural proteins (p7 through NS5A/B) (3,4). Many of the

nonstructural proteins are essential for productive viral replication (5).

The development of selectable subgenomic HCV RNA replicons has led to important advances in the field of HCV. Recently, several groups have reported the full-length infectious tissue culture system (6,7,8), which is likely to further our understanding of the infectious process. The subgenomic replicons are bicistronic constructs composed of the HCV IRES, the neomycin phosphotransferase (neo) gene, and the encephalomyocarditis IRES, which mediates the translation of the HCV nonstructural proteins NS3 through NS5, followed by the 3'NCR (9).

Chronic HCV is characterized by several histological features of the liver such as bile duct damage, lymphoid follicles, low serum cholesterol levels and in about 50-60% of the cases steatosis (10,11). Recently genomic analysis conducted on livers of HCV infected chimpanzees revealed that transcript levels of genes involved in lipid metabolism and homeostasis are altered (12,13).

The liver is a major site of lipid synthesis. Many of the synthesized lipids are exported from the liver in the form of apolipoprotein containing particles known as very-low-density-lipoproteins (VLDLs), precursors of low-density lipoproteins (LDL). The VLDLs are composed of a nonpolar core of triglycerides and cholesteryl esters surrounded by a monolayer amphipathic coating of protein, phospholipids and cholesterol (14,15). VLDLs are formed in the luminal space within the endoplasmic reticulum (ER) while the lipids that are required for their formation are synthesized on the cytosolic side of the ER membrane (16). The

ER is also the center of HCV translation and replication activities, resulting in the ER stress response and in the induction of oxidative stress (17).

The microsomal triglyceride transfer protein (MTP) and apolipoprotein B100 (apoB) are major regulators of VLDL assembly in the liver and have been shown to physically interact during VLDL assembly (18-22). ApoB is the major structural protein associated with the VLDLs and LDLs (23). In the presence of sufficient lipid, mainly triglycerides, apoB is efficiently assembled into secretion-competent VLDL particles (23). If, however, apoB is poorly lipidated the majority of newly synthesized apoB is degraded by the proteasome in the cytosol (24) or by ER-resident proteases (25,26). MTP stabilizes apoB by lipidation and subsequently lipidated apoB fuses with triglyceride-rich particles, leading to the formation of VLDLs (27,28). MTP is present in the ER lumen as a heterodimeric protein consisting of the active 97 kDa monomer linked with a protein disulfide isomerase (29). It transfers lipids from various donor lipid sites to acceptor sites and plays a fundamental role in the cotranslational lipidation of apoB as it enters the ER lumen, thus preventing degradation (28). Although MTP is capable of transferring all classes of lipid found in apoB lipoproteins (triglyceride, cholesteryl esters, free cholesterol and phospholipids), *in vitro* analyses show that it strongly prefers triglycerides and cholesteryl esters (30).

During the early stages of HCV infection, host genes involved in lipid metabolism are differentially regulated (12). HCV infection causes the formation of hepatocellular lipid droplets onto which HCV structural and nonstructural proteins have been shown to colocalize (12). In addition, HCV core protein induces steatosis by itself and colocalizes with the cytoplasmic globular lipid structures (31). HCV core protein also reduces the activity of MTP (31). Even though core can associate with several cellular proteins, direct association of HCV core protein with apoB or MTP has not been demonstrated (32). In addition, the HCV nonstructural protein NS5A colocalizes with core protein on lipid droplets and interacts with apolipoprotein AI and AII (protein components of high-density lipoproteins) (33). In chronic HCV infection, hypobetalipoproteinemia

is prevalent especially in patients infected with genotype 3 (34).

In this study, we examine the role of the HCV nonstructural proteins on lipid export via apoB using the HCV subgenomic replicon system. Secretion of apoB via lipoproteins was drastically reduced in the cells containing the HCV subgenomic replicon while in cell lines cured of the replicon, apoB secretion was restored. A potential reason for the decrease in apoB secretion could be due to the lower triglyceride levels that we detected. Our studies further revealed that MTP activity was decreased in the subgenomic replicon containing cells. In addition, MTP transcript levels and promoter activity were reduced. These results provide evidence that HCV subgenomic replicons inhibit lipoprotein secretion by interfering with the formation of secretion-competent apoB lipoproteins via inhibition of MTP. Moreover, apoB is shown here to interact with the HCV nonstructural protein NS5A suggesting that apoB may be a target for HCV in the context of the HCV subgenomic replicon.

MATERIALS AND METHODS

Cell Culture, Immunoprecipitations and Reporter assays - Huh-7, FCA4 (gift of C. Seeger, Institute for Cancer Research, Fox Chase Center, PA) and K2040 (gift of Michael Gale, UT Southwestern) human hepatoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Huh-7.5 cured cells were a gift from C. Rice at Rockefeller U. The K2040 cured cells (KC) were cured as described by Tardif et al (35). For metabolic labeling, Huh-7, FCA4 and K2040 cells (and the cured cells) were labeled for the indicated times with 100 μ Ci of Trans ³⁵S label (specific activity 1,175 Ci/mmol; MP Biomedicals, Inc.) per ml of the above medium. In some experiments 40 μ g/ml of N-acetyl-leucyl-leucyl-norleucinal (ALLN) were added for 4 hr prior to the labeling. Cellular lysates and media were immunoprecipitated with goat anti-apolipoprotein B polyclonal antibody (Chemicon International) or rabbit anti human-albumin antibody (ICN Biomedicals, Inc) at 4°C for 16 hrs. The immune complexes were analyzed by SDS-PAGE using a 4-15% SDS-PAGE gradient gel

(Biorad). For pulse-chase experiments, indicated cells were labeled with Trans ³⁵S label and were chased over various time points in complete DMEM. Cells and chase medium were collected, and immunoprecipitated as stated above. ALLN (40 μg/ml) was added to the medium in some cases prior to the pulse as well as during the chase periods. Oleic acid conjugated to BSA (30 μl per ml of media) (Sigma) was added to the medium 24 hrs prior to the pulse in some cases. For transfections, cells at ~70% confluency were transfected using Lipofectamine 2000 reagent (Invitrogen). For protein-protein interaction studies, indicated cellular lysates were prepared as described previously (36). Lysates were immunoprecipitated with anti-apoB antibody followed by immunoblotting with anti NS5a (gift of C. Cameron, Pennsylvania State University) and anti-NS4a (Virogen). The HCV NS5A coding sequence and the NS5A mutants were generated as described by Gong et al (37). For MTP promoter-luciferase reporter (gift of R. Davis, 38) assays, lysates were assayed as described previously (36). A -gal expression plasmid was used as an internal control for transfection.

Overall Triglyceride Levels - Triglycerides were quantitated using the L-Type TG H kit (Wako Chemicals USA, Inc) according to the manufacturer's protocol. Briefly, 3.68x10⁶ cells per reaction (Huh-7, FCA4 and K2040 cells) were collected. Lipids were extracted as described previously (39) and then resuspended in 200 μl of 2% triton X-100 in PBS. 40 μl of lipids were added to 533.3 μl of enzyme color A, incubated at 37°C, 5 minutes. 266.6 μl of color enzyme B were then added and the samples incubated for 5 minutes at 37°C. The absorbance was then read at 595 nm. The reactions were standardized using lipid calibrator (Wako Chemicals USA, Inc). Triplicate reactions were carried out with each cell line.

Measurement of MTP Activity - Huh-7, FCA4 and K2040 cellular lysates were divided into 250 μl aliquots and sonicated on ice. Proteins were quantitated by Bradford assay. MTP activity was determined using the MTP activity kit (Roar Biomedical, New York, NY) according to the manufacturer's protocol. Briefly, 150 μg of protein were combined with 6 μl each of suspensions of donor and acceptor particles. The

change of fluorescent intensity was monitored (excitation at 465 nm and emission at 535 nm). The reaction was allowed to proceed over a 720 min time course.

Quantitative Real-time PCR - Total RNA was extracted using RNA STAT-60 reagent (TEL-TEST, Inc.) according to manufacturer's standard procedures. 1 μg of total RNA was preincubated with oligo(dT) primer (Invitrogen) at 70°C for 5 min and then on ice for 5 min, then used to generate a cDNA library using ImProm-II reverse transcriptase (Fisher). 1.5μl of cDNA library were added to each 25μl PCR reaction using Quantitect SYBR Green PCR reagent (QIAGEN). Quantifications were done in triplicate and repeated three separate times. Amplification reactions were performed using primers for either MTP: 5'-GGAGCTTCCCCAAGAAATGAAT-3' (MTP-FWD), 5'-GAACTCGACGGACAATTTTGTCT-3' (MTP-REV) or for hypoxanthine-phosphoribosyltransferase (HPRT): 5'-TTGGTGGAGATG-ATCTCTCAAC-3' (HPRT-FWD), 5'-AGCTTGCGACCTTGACCAT-3' (HPRT-REV). Relative transcript levels were calculated using the ΔΔCt method as specified by the manufacturer.

RESULTS

HCV subgenomic replicon decreases intact synthesis and secretion of apoB - To study the effect of the HCV nonstructural proteins on apoB synthesis and secretion we first set out to determine if we could detect apoB synthesis and secretion in the HCV subgenomic replicon containing cells. Huh-7 cells have been shown to secrete lipoprotein particles that contain apoB100 (16). ApoB synthesis and secretion were determined by metabolically labeling Huh-7 cells (control) and FCA4 cells (HCV replicon containing cells) with ³⁵S-methionine followed by immunoprecipitation (IP) with an apolipoprotein B antibody. The immunoprecipitates were then subjected to SDS-PAGE. The results show intact apoB (fully lipidated; 550 kDa) in both the lysates (Fig. 1A lanes 1 and 2). In order to detect apoB secretion out of the cells via lipoproteins, ³⁵S-methionine labeled Huh-7 and FCA4 culture media were immunoprecipitated with the anti-apoB antibody.

Intact apoB was detected in the Huh-7 medium while there was no evidence for apoB secretion into the FCA4 culture medium (Fig. 1A, lanes 3 and 4). These results indicate that apoB synthesis occurs in Huh-7 as well as the FCA4 cells. However, apoB secretion was significantly decreased in HCV expressing FCA4 cells.

To determine if our observations are due to clonal differences between the parental Huh-7 cells and the Huh-7 cells harboring the HCV replicon, cured replicon cells (cured by prolonged treatment with interferon (IFN- α)) were also examined by the same method as above (Fig. 1B). Two separate cured cell lines (Huh-7.5 and K2040 cured (KC)) were both able to restore apoB secretion into the media (Fig. 1B lanes 3-6). Consequently, the decrease in apoB secretion can be attributed to the presence of the HCV replicon.

To further analyze apoB synthesis, we pulse-labeled Huh-7, FCA4 and K2040 cells with ^{35}S -methionine from 15 min to 60 min followed by an immunoprecipitation with antibodies against either apoB or albumin. K2040 is another HCV subgenomic replicon containing cell line that was used to rule out possible clonal effects of FCA4. At each time point, there were lower levels of intact apoB in the FCA4 and K2040 cells compared to the levels in the Huh-7 cells (Fig. 1C upper panel). In addition, albumin protein synthesis remained constant at the various time points among all three cell lines indicating a specific inhibitory effect on apoB synthesis in the presence of the HCV subgenomic replicon (Fig. 1C lower panel).

Secretion level of apoB was decreased in the presence of the HCV nonstructural proteins - Lipoprotein secretion is reduced when synthesis of apoB is inhibited or when degradation of apoB is elevated due to a lack of lipidation (40-42). To demonstrate the secretion rate of apoB100 in the presence of the HCV subgenomic replicon Huh-7, FCA4 and K2040 cells were pulse-labeled with ^{35}S -methionine and then chased for up to 2 hrs. The cell lysates as well as the culture media were monitored for intact apoB as well as albumin by immunoprecipitation using anti-apoB or anti-albumin antibodies. We detected slightly lower levels of apoB in the FCA4 and K2040 cellular lysates compared to the Huh-7 cellular lysates (Fig. 2 cellular panels). However, the secretion rate of apoB was dramatically reduced in the

FCA4 and K2040 cells compared to the secretion rate in the Huh-7 cells (Fig. 2 secreted panels). We were able to detect a small amount of apoB secretion in the FCA4 and K2040 cells at later chase times indicating that apoB secretion can occur in these cells, but at considerably lower levels (Fig. 2). Also, general protein secretion was not affected in the replicon containing FCA4 and K2040 cells, as determined by the secretion of albumin into the culture media (Fig. 2 secreted albumin panels).

The degradation of apoB due to poor lipidation occurs via the proteasome in the cytosol and by proteases in the endoplasmic reticulum (16). ALLN, a cysteinyl protease inhibitor has been shown to help block both of these activities (43). We tested whether apoB secretion would increase in the replicon containing cells if apoB degradation were inhibited. Huh-7, FCA4 and K2040 cells were pulse-labeled with ^{35}S -methionine in the presence of ALLN, chased for up to 2 hrs and then immunoprecipitated with anti-apoB or anti-albumin antibodies. We were able to detect synthesized intact apoB in all the cellular lysates in the presence of ALLN indicating that ALLN does not inhibit apoB synthesis (Fig. 3 cellular panels). ApoB secretion by FCA4 and K2040 cells was not increased when degradation of apoB was inhibited by ALLN (Fig. 3 secreted apoB panels). These cells were, however, able to secrete albumin at similar levels to Huh-7 cells (Fig. 3 secreted albumin panels). These results suggest that degradation of apoB is not the major reason for the inability of HCV replicon containing cells to secrete apoB into the culture medium.

Triglyceride levels decreased in the presence of the HCV subgenomic replicon - ApoB synthesis and ultimate secretion as part of a lipoprotein complex depends upon proper lipidation of apoB, in particular, by triglycerides. Using a colorimetric assay we determined the amount of triglycerides in Huh-7, FCA4 and K2040. We detected around a 30% reduction of triglycerides in the replicon containing cells compared to Huh-7 cells (Fig. 4). The replicon containing cells therefore have less triglycerides available to lipidate apoB for secretion.

Oleic acid treatment does not restore apoB secretion. We examined the effect of oleic acid treatment of the Huh-7 cells, the replicon

containing cells and the cured cells on apoB secretion. Following treatment of the cells with oleic acid for 24hrs, the cells were pulse-labeled with ³⁵S-methionine followed by immunoprecipitation with either anti-apoB antibody or anti-albumin antibody. As we have shown before without oleic acid treatment (Fig. 1), there were slightly lower levels of synthesized apoB in the replicon containing cellular lysates compared to the Huh-7 and cured cellular lysates (Fig. 5). More importantly however, there was no evidence of restored apoB secretion in the replicon containing cells, while albumin secretion was similar in all the cell lines (Fig. 5). These results indicate that even in the presence of increased levels of free fatty acids, which should increase the amount of triglycerides available for lipoprotein synthesis and secretion, apoB secretion is not restored in the HCV subgenomic replicon containing cells.

HCV subgenomic replicon reduces MTP activity, transcript levels and promoter activity - MTP transports the lipids from the ER lumen to the growing apoB polypeptide chain in the lumen of the ER, allowing proper translocation and folding to occur (44). In addition, MTP is also necessary for the formation of protein-free triglyceride droplets in the ER-lumen that fuse with the apoB containing particles prior to secretion as lipoproteins (45). Using the subgenomic replicon system we determined the effect of the HCV nonstructural proteins on MTP in the absence of core protein. An MTP activity assay was performed on Huh-7, FCA4 and K2040 cellular lysates. There was an observable reduction in MTP activity in the FCA4 and K2040 cellular lysates (Fig. 6A). In addition MTP transcript levels determined by quantitative RT-PCR were about 80-96% lower in the K2040 and FCA4 cells respectively (Fig. 6B). A luciferase assay using an MTP-luciferase promoter construct revealed that in the presence of the HCV nonstructural proteins MTP promoter activity was reduced 74% in the K2040 cells and 94% in the FCA4 cells compared to the Huh-7 cells (Fig. 6C). In a transient transfection assay Huh-7 cells were transfected with the HCV subgenomic replicon RNA (BM4-5) or with a control RNA. The MTP promoter activity levels were reduced 40% in the BM4-5 transfected cells compared to the control cells (Fig. 6D). Interestingly, in the cured cell line Huh-

7.5, MTP promoter activity was found to be slightly greater than Huh-7 cells expressing wild-type MTP promoter activity levels (data not shown). These results suggest that MTP activity, transcript level, and promoter activity are reduced in the presence of the HCV nonstructural proteins. This reduction will severely impact the lipidation of apoB by triglycerides in the cells and therefore cause a decrease in apoB secretion.

HCV nonstructural protein, NS5A, interacts with apoB - The HCV nonstructural protein NS5A has been shown to be capable of interacting with various components of cellular lipid metabolic pathways (33). NS5A not only localizes to the ER and Golgi apparatus but also colocalizes with the HCV core protein on cytoplasmic lipid droplets (33). To determine if NS5A interacts with apoB, Huh-7 cells were transiently transfected with an NS5A expression vector. The cellular lysates were then immunoprecipitated with anti-apoB antibody and subsequently immunoblotted with anti-NS5A serum. The results show the interaction of NS5A with apoB (Fig. 7A, lanes 1 and 2). To confirm this interaction in the context of the HCV subgenomic replicon, cellular lysates from FCA4 and K2040 cells were immunoprecipitated with anti-apoB antibody followed by immunoblotting using an anti NS5A serum (Fig. 7A, lanes 3 and 4). In addition, cellular lysates from FCA4 and K2040 cells were treated the same as above except immunoblotting was done using anti NS4 antibody. No interaction was detected (data not shown). These results confirm the interaction between NS5A and apoB in the context of the subgenomic replicon.

We further analyzed this interaction by mapping the apoB binding region of NS5A. We transiently transfected Huh-7 cells with NS5A deletion mutants (Fig. 7B, bottom panel). We only detected an interaction with mutant 1 which contains the C-terminal region of NS5A (Fig. 7B). This indicates that the C-terminal portion of NS5A may be important for binding, however it may require a certain conformation for binding since there was no binding with mutant 2, which contains a smaller portion of the NS5A C-terminal region.

DISCUSSION

Alteration of lipid metabolism has been shown to occur in the presence of HCV gene expression. Multiple cellular genes involved in lipid metabolism are differentially regulated in HCV acutely infected chimpanzees (12,13). In animal models, the HCV core protein is steatogenic when expressed in the liver of mice (46). In this study using the HCV subgenomic replicon system, we have determined that the HCV nonstructural proteins NS3-NS5B, in the absence of core protein, are able to alter lipid metabolism and in particular are able to reduce the activity and the transcript and the protein levels of MTP, a major regulator of the assembly and secretion of triglyceride-rich lipoprotein particles. In addition we have determined that one of the HCV nonstructural proteins, NS5A, interacts with apoB.

Interestingly, steatosis occurs when there is an accumulation of triglycerides. We, however, observed a modest decrease in triglyceride synthesis in replicon expressing cells. Steatosis linked to HCV has been demonstrated to be much more prevalent in genotype 3 infections compared to genotype 1 in European countries (47-51). However in Japan hepatic steatosis is common in patients with 1b HCV infection (52). It has been suggested that there may be some differences in the amino acid sequences in the genotype 1b HCV clones between Japan and European countries (53). The HCV subgenomic replicons that were used in this study represent genotype 1b described by Guo et al (54) that represents the European 1b genotype. In addition, *in vitro* and *in vivo* studies have shown that HCV core protein can induce steatosis in transfected cells (55) and transgenic mice (56). Core protein is absent in the HCV subgenomic replicon containing cells. This study demonstrates that HCV subgenomic replicon expression is capable of altering lipid metabolism in cultured cells.

We have developed a system to monitor apoB synthesis and secretion in the presence of the HCV subgenomic replicon. Using this system we detected slightly reduced levels of intact apoB synthesis in the replicon containing cells. We also determined that in these same cells the secretion of apoB was very low. This effect is specific to the presence of the active HCV subgenomic replicon since apoB secretion does occur efficiently in cured cell lines. Since apoB degradation occurs when apoB is not fully lipidated by triglycerides,

we specifically monitored apoB secretion in the presence of ALLN, a cysteinyl protease inhibitor that has been previously determined to help block apoB degradation. Even when apoB degradation was reduced in the replicon cells, secretion of apoB did not occur. Some evidence suggests that apoB is produced in excess of the needs for VLDL secretion and that at least 70% of synthesized apoB undergoes intracellular proteolytic degradation (57). In our experiments we always detected lower amounts of secreted apoB relative to synthesized levels of apoB. The addition of ALLN alone did not change the overall synthesis and secretion of apoB in the replicon containing cells indicating that degradation of apoB is not the major cause for the lack of apoB secretion in these cells. Our results also correlate well with a recent study conducted on the serum lipids of patients with genotype 1b HCV infection in Japan, in which serum apoB levels were significantly reduced compared to patients infected with HBV or HCV genotype 2a (53).

Lipidation of apoB by triglycerides is regulated at least at three independent steps: synthesis of triglycerides, transfer of triglycerides across the ER membrane and delivery of triglycerides from the ER membrane to apoB by MTP (16). The process and regulation of the transfer of triglycerides across the ER membrane is not well understood (16). In the presence of the HCV subgenomic replicon we have detected a decrease in triglyceride levels. Lower triglyceride levels do help to explain the reduction in apoB synthesis and secretion detected. However, why we are seeing this decrease in triglycerides is difficult to determine. The decrease in triglyceride levels is small and therefore the impact on overall apoB secretion is also small and is not believed to be the major cause for the lower levels of apoB secreted. In addition, when oleic acid was added, apoB secretion could not be restored in the replicon expressing cells suggesting that the decrease in apoB secretion is likely due to low MTP activity rather than a lack of available triglycerides. Further investigation is currently underway to better understand the impact free fatty acids have on apoB secretion in the context of the HCV subgenomic replicon.

The production by the liver of apoB containing lipoproteins is also regulated by the relative amount of *de novo* synthesized apoB that is

translocated into the ER and assembled into a secretion-competent lipoprotein (44,58-60). The rate of VLDL secretion by the liver is closely linked to the expression and activity of MTP (61-63). HCV core protein inhibits the MTP-mediated transfer of triglycerides to the growing nascent apoB via an unknown mechanism (65). Our results provide evidence that the expression of the HCV nonstructural proteins from the HCV subgenomic replicon reduces MTP activity providing evidence for the reduction in apoB secretion in these cells. Since apoB is secreted as a component of triglyceride-rich lipoprotein particles, inhibition of MTP would therefore decrease the addition of triglycerides to apoB containing particles thus reducing secretion. In addition, unlike core, which does not appear to impact MTP expression levels and has no direct association with MTP or apoB, we have demonstrated that the expression of the subgenomic replicon leads to dramatic reduction of the transcript levels as well as the promoter activity of MTP. It is possible that the repression of the MTP promoter is not due to direct association with an HCV nonstructural protein but involves the interaction of a nonstructural protein(s) with other factors involved in MTP transcription. This is supported by the observations that in the cured HCV cell lines the MTP promoter activity is restored demonstrating the regulatory role of HCV nonstructural proteins in the observed phenomenon.

The HCV nonstructural protein NS5A is a multifunctional phosphoprotein that is believed to have various functions in HCV replication, interferon resistance and HCV pathogenesis

(4,33). It has recently been determined that NS5A associates with apolipoproteins AI and AII (33,55). These apolipoproteins regulate hepatic lipase-mediated hydrolysis of triglycerides and phospholipids in high-density lipoprotein (HDL) particles. In addition, previous studies by others have suggested an association of NS5A with the ER and the Golgi apparatus (33). NS5A co-localizes with HCV core protein on cytoplasmic lipid droplets (33). We have now further implicated NS5A in contributing to altered lipid metabolism by establishing an interaction between NS5A and apoB at the c-terminal region of NS5A. At this time it is not known if this interaction is direct or indirect via other proteins or lipids however we were able to detect this interaction in the absence of other HCV proteins and we were not able to detect any interaction between apoB and NS4 in FCA4 and K2040 cells. The interaction of apoB and NS5A does establish a connection between lipid secretion and HCV nonstructural proteins in the context of the subgenomic replicon.

In summary, this study demonstrates the ability of HCV NS proteins to alter the course of apoB synthesis, assembly and secretion pathways in Huh-7 cells. At the cellular level, there was a significant and notable reduction of apoB secretion while at the molecular level MTP mRNA and MTP activity was greatly diminished. The results shed light on the potential role of HCV gene expression in modifying lipid export and contribute to the spectrum of liver disease pathogenesis associated with chronic hepatitis C.

REFERENCES

1. Giannini, C., and Brechot, C. (2003) *Cell Death Diff.* **10**, S27-S38
2. Di Biscegli, A.M. (1997) *Hepatology* **26** (3 suppl. 1), 34S-38S
3. Bartenschlager, P., and Lohmann, V. (2000) *J. Gen. Virol.* **81**, 1631-1684
4. Dubuisson, J., Penin, F., and Moradpour, D. (2002) *Trends Cell Biol.* **12**, 517-523
5. Kolykhalov, A. A., Mihalik, K., Feinstone, S. M., and Rice, C. M. (2000) *J. Virol.* **74**, 2046-2051
6. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H., Mizokami, M., Bartenschlager, R. and Liang, T. J. (2005) *Nature Med.* **11**, 791-796
7. Lindenbach, B. D., Evans, M.J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C.C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A., and Rice, C. M. (2005) *Science* **309**(5734), 623-626

8. Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T., and Chisari, F. V. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 9294-9299
9. Lohmann, V., Korner, F., Koch, J.-O., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) *Science* **285**, 110-113
10. Fujie, H., Yotsuyanagi, H., Moriya, K., Shintani, Y., Tsutsumi, T., Takayama, T., Makuuchi, M., Matsuura, Y., Miyamura, T., Kimura, S., and Koike, K. (1999) *J. Med. Virology* **59**, 141-145
11. Kyoji, M., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Yotsuyanagi, H., Lino, S., Kimura, S., and Koike, K. (2003) *Hepatology Res.* **25**, 371-376
12. Su, A. I., Pezacki, J. P., Woodiccka, L., Brideau, A. D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G., and Chisari, F. C. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 15669-15674
13. Bigger, C. B., Guerra, B., Brasky, K. M., Hubbard, G. Beard, M. R., Luxon, B. A., Lemon, S. M., and Lanford, R. E. (2004) *J. Virol.* **78**, 13779-13792
14. Fisher, E. A., and Ginsberg, H. N. (2002) *J. Biol. Chem.* **277**, 17377-17380
15. Rustaeus, S., Lindberg, K., Stillemark, P., Claesson, C., Asp, L., Larsson, T., Boren, J., Olofsson, S.O. (1999) *J. Nutr.* **129**, 463S-466S
16. Higashi, Y., Itabe, H., Fukase, H., Mori, M., Fujimoto, Y., and Tankano, T. (2003) *J. Biol. Chem.* **278**, 21450-21458
17. Tardif, K.D., Waris, G., and Siddiqui, A. (2005) *Trends Microbiol.* **13**, 135-163
18. Andre, P., Perlemuter, G., Budkowska, A., Brechot, C., and Lotteau, V. (2005) *Seminars in Liver Disease* **25**, 93-104
19. Wu, X., Zhou, M., Huang, L. S., Wetterau, J., and Ginsberg, H. N. (1996) *J. Biol. Chem.* **271**, 10277-10281
20. Patel, S. B., and Grundy, S. M. (1996) *J. Biol. Chem.* **271**, 18686-18694
21. Hussain, M. M., Bakillah, A., Nayak, N., and Shelness, G. S. (1998) *J. Biol. Chem.* **273**, 25612-25615
22. Bakillah, A., Jamil, H., and Hussain, M. M. (1998) *Biochemistry* **37**, 3727-3734
23. Adeli, K. (1994) *J. Biol. Chem.* **269**, 9166-9175
24. Yeung, S. J., Chen, S. H., and Chan, L. (1996) *Biochemistry* **35**, 13843-13848
25. Adeli, K., Wettsten, M., Asp, L., Mohammadi, A., Macri, J., and Olofsson, S.-O. (1997) *J. Biol. Chem.* **272**, 5031-5039
26. Wu, X., Sakata, N., Lele, K. M., Zhou, M., Jiang, H., and Ginsberg, H. N. (1997) *J. Biol. Chem.* **272**, 11575-11580
27. Gordon, D. A. (1997) *Curr. Opin. Lipidol.* **8**, 131-137
28. Raabe M., Veniant, M. M., Sullivan, M. A., Zlot, C. H., Bjorkegren, J., Nielsne, L. B., Wong, J. S., Hamilton, R. L., Young, S. G. (1999) *J. Clin. Invest.* **103**, 1287-1298
29. Gordon, D. A., and Jamil, H. (2000) *Biochim. Biophys. Acta* **1486**, 72-83
30. Jamil, H., Dickson Jr., J. K., Chu, C.-H., Lago, M. W., Rinehart, J. K., Biller, S. A., Gregg, R. E., and Wetterau, J. R. (1995) *J. Biol. Chem.* **270**, 6549-6554
31. Negro, F. (2002) *Hepatology* **36**, 1050-1052
32. Sabile, A., Perlemuter, G., Bono, F., Kohara, K., Demaugre, F., Kohara, M., Matsuura, Y., Miyamura, T., Brechot, C., Barba, G. (1999) *Hepatology* **30**, 1064-1076
33. Shi, S. T., Polyak, S. J., Tu, H., Taylor, D. R., Gretch, D. R., and Lai, M. M. C. (2002) *Virol.* **292**, 198-210
34. Serfaty, L., Andreani, T., Giral, P., Carbonell, N., Chazouilleres, O., Poupon, R. (2001) *J. Hepatology* **34**, 428-434
35. Tardif, K.D., Mori, K., Kaufman, R.J., and Siddiqui, A. (2004) *J. Biol. Chem.* **279**, 17158-17164
36. Waris, G., and Siddiqui, A. (2005) *J. Virol.* **79**, 9725-9734
37. Gong, G., Waris, G., Tanveer, R., and Siddiqui, A. (2001) *Proc. Natl. Acad. Sci.* **98**, 9599-9604
38. Kang, S., Spann, N.J., Hui, T.Y., and Davis, R. A. (2003) *J. Biol. Chem.* **278**, 30478-30486
39. Folch, J., Lees, M., and Slone-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497-509

40. Yeung, S. J., Chen, S.H., and Chan, L. (1996) *Biochemistry* **35**, 13843-13848
41. Fisher, E.A., Zhou, M., Mitchel, D. M., Wu, X., Omura, S., Wang, H., Goldberg, A. L., and Ginsberg, H. N. (1997) *J. Biol. Chem.* **272**, 20427-20434
42. Adeli, K., Macri, J., Mohammadi, A., Kito, M., Urade, R., and Cavallo, D. (1997) *J. Biol. Chem.* **272**, 22489-22494
43. Cavallo, D., Rudy, D., Mohammadi, A., Macri, J., and Adeli, K. (1999) *J. Biol. Chem.* **274**, 23135-23143
44. Davidson, N. O., and Shelness, G. S. (2000) *Annu. Rev. Nutr.* **20**, 169-193
45. Shelness, G. S., and Sellers, J. (2001) *Curr. Opin. Lip.* **12**, 151-157
46. Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., Koike, K. (1997) *J. Gen. Virol.* **78**, 1527-1531
47. Kumar, D., Farrell, G. C., Fung, C., and George, J. (2002) *Hepatology* **36**, 1266-1272
48. Pawlowsky, J. M., Tsakiris, L., Roudot-Thoraval, F., Pellet, C., Stuyver, L., Duval, J., Dhumeaux, D. (1999) *J. Infect. Dis.* **171**, 1607-10
49. Rubbia-Brandt, L., Quadri, R., Abid, K., Giostra, E., Male, P. J., Mentha, G., Spahr, L., Zarski, J. P., Borisch, B., Hadengue, A., Negro, F. (2000) *J. Hepatol.* **33**, 106-15
50. Mihm, S., Fayyazi, A., Hartman, H., and Ramadori, G. (1997) *Hepatology* **25**, 735-739
51. Sefaty, L., Andreani, T., Giral, P., Carbonell, N., Chazoilleres, O., and Poupon, R. (2001) *J. Hepatol.* **34**, 428-34
52. Fujie, H., Yotsuyanagi, H., Moriya, K., Shintani, Y., Tsutsumi, T., Takayama, T., Makuuchi, M., Matsuura, Y., Miyamura, T., Kimura, S., Koike, K. (1999) *J. Med Virol.* **59**, 141-145
53. Moriya, K., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsuni, T., Yotsuyanagi, H., Iino, S, Kimura, S., and Koike, K. (2003) *Hepatology Res.* **25**, 371-376
54. Guo, J., Bichko, V. V., and Seeger, C. (2001) *J. Virol.* **75**, 8516-8523
55. Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y, Eder, G., Schaff, A., Chapman, M. J., Miyamura, T., and Brechpt, C. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1200-1205
56. Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., Koike, K. (1997) *J. Gen. Virol.* **78**, 1527-1531
57. Adeli, K., Taghibiglou, C., Van Inderstine, S. C., Lewis, G. F. (2001) *Trends Cardiovasc. Med.* **11**, 1170-1176
58. Davis, R. A. (1999) *Biochim. Biophys. Acta* **1441**, 1-31
59. Olofsson, S. O., Asp, L., and Boren, J. (1999) *Curr. Opin. Lipidol.* **10**, 341-346
60. Fisher, E. A., and Ginsberg, H. N. (2002) *J. Biol. Chem.* **277**, 17377-17380
61. Raabe, M., Flynn, L. M., Zlot, C. H., Wong, J. S., Veniant, M. M., Hamilton, R. L., and Young, S. G. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8686-8691
62. Jamil, H., Chu, C. H., Dickson, J. K., Jr., Chen, Y., Yan, M., Biller, S. A., Gregg, R. E., Wetterau, J. R., and Gordon, D. A. (1998) *J. Lipid Res.* **39**, 1448-1454
63. Liao, W., Kobayashi, K., and Chan, L. (1999) *Biochemistry* **38**, 7532-7544
64. Tietge, U. J., Bakillah, A., Maugeais, C., Tsukamoto, K., Hussain, M., and Rader, D. J. (1999) *J. Lipid Res.* **40**, 2134-2139
65. Perlemuter, G., Sabile, A., Letteron, P., Vona, G., Toplico, A., Chretien, Y., Koike, K., Pessayre, D., Chapman, J., Barba, G., Brechot, C. (2002) *FASEB J.* **16**, 185-194

FOOTNOTES

* We thank Lisa Kuelzo for assistance with MTP activity assay and Jerome Schaack for critical review of the manuscript. A.D was supported by the W.M. Thorkildsen Research award. This work is supported by an NIH grant DK061566 awarded to A.S.

¹The abbreviations used are: apoB, apolipoprotein B100; ALLN, N-acetyl-leucyl-leucyl-norleucinal; MTP, microsomal triglyceride transfer protein; HCV, hepatitis C virus; VLDL, very low density

lipoprotein; LDL, low density lipoprotein; IRES, internal ribosomal entry site; ER, endoplasmic reticulum; HBV, hepatitis B virus; IFN, interferon.

FIGURE LEGENDS

Fig. 1. Detection of apoB in Huh-7 and HCV subgenomic replicon cells. *A*, detection of apoB. Huh-7 and FCA4 (contains HCV subgenomic replicon) cells were radiolabeled with ^{35}S for 1 hr. Solubilization buffer was added to the cells as well as to the culture medium. Both the cells (cellular) and medium (secreted) were then immunoprecipitated with anti-apoB antibody. The immunoprecipitated samples were subjected to SDS-PAGE and autoradiography. H (Huh-7); F (FCA4). *B*. Detection of apoB in cured cells. Huh-7 and the cured cells Huh-7.5 and K2040C were treated as above. 7.5 (Huh-7.5); KC (K2040C). *C*, effect of HCV on the synthesis of apoB. Huh-7, FCA4 and K2040 cells were radiolabeled with ^{35}S for 15, 30 or 60 mins as indicated. The cells were lysed in solubilization buffer then immunoprecipitated with either anti-apoB antibody or with anti-albumin antibody as a control.

Fig. 2. ApoB secretion is lower in HCV subgenomic replicon cells. Effect of HCV on apoB secretion rates. Huh-7, FCA4, K2040 cells were pulse-labeled with ^{35}S for 20 mins and chased for the indicated times. The cells were lysed and the lysates and medium were immunoprecipitated as stated in Fig. 1.

Fig. 3. ApoB secretion is not increased when apoB degradation is inhibited in HCV subgenomic replicon cells. Huh-7, FCA4 and K2040 cells were treated with ALLN for 4 hrs and then pulse-labeled for 20 mins and chased for the indicated times. The cells and media were then treated and immunoprecipitated as for Fig. 1.

Fig. 4. Overall triglyceride levels. Lipids extracted from Huh-7, FCA4, and K2040 cells underwent a colorimetric assay to determine the amount of triglycerides per cell line. The values are the averages and standard deviations of three separate experiments.

Fig. 5. ApoB secretion is not restored in the presence of oleic acid. Huh-7 (H), FCA4 (F), K2040 (K), Huh-7.5 (7.5) and cured K2040 (KC) cells were treated with oleic acid for 24 hrs and then pulse-labeled with ^{35}S for 1 hr. Cells were then treated as stated in Fig. 1.

Fig. 6. MTP activity, transcript level and promoter activity reduced in presence of HCV subgenomic replicon. *A*, MTP activity in Huh-7 (diamonds), FCA4 (squares) and K2040 (triangles) homogenates was assayed. 150 μg of protein were combined with suspended donor and acceptor particles and incubated at 37°C for up to 720 min. During the incubation, the change of fluorescent intensity was monitored. The values represent the averages and standard deviations from three independent experiments. *B*, QT-RT-PCR was done to monitor the transcript levels of MTP in Huh-7, K2040 and FCA4 cells. The values are the average and standard deviations of three separate experiments. *C*, MTP promoter activity was assayed in Huh-7, K2040 and FCA4 cells using an MTP luciferase reporter assay. The results are plotted as the luciferase activity divided by the beta-galactosidase activity. Values are the averages and standard deviations from triplicate reactions. *D*, MTP promoter activity was assayed in Huh-7 cells transiently transfected with either control RNA (truncated BM4-5) or BM4-5 RNA (HCV subgenomic replicon RNA). The results are plotted as above.

Fig. 7. NS5A interacts with apoB. *A*, Huh-7 cells were transiently transfected with and without NS5A expression plasmid. Cellular lysates from these cells and FCA4 and K2040 cells were then immunoprecipitated with apoB antibody followed by a western blot assay using an anti-NS5A antibody as described in 'Materials and Methods'. *B*, Huh-7 cells were transiently transfected with the NS5A

expression plasmid or with one of the NS5A deletion mutant expression plasmids (bottom of figure) and then immunoprecipitated and western blotted as above.

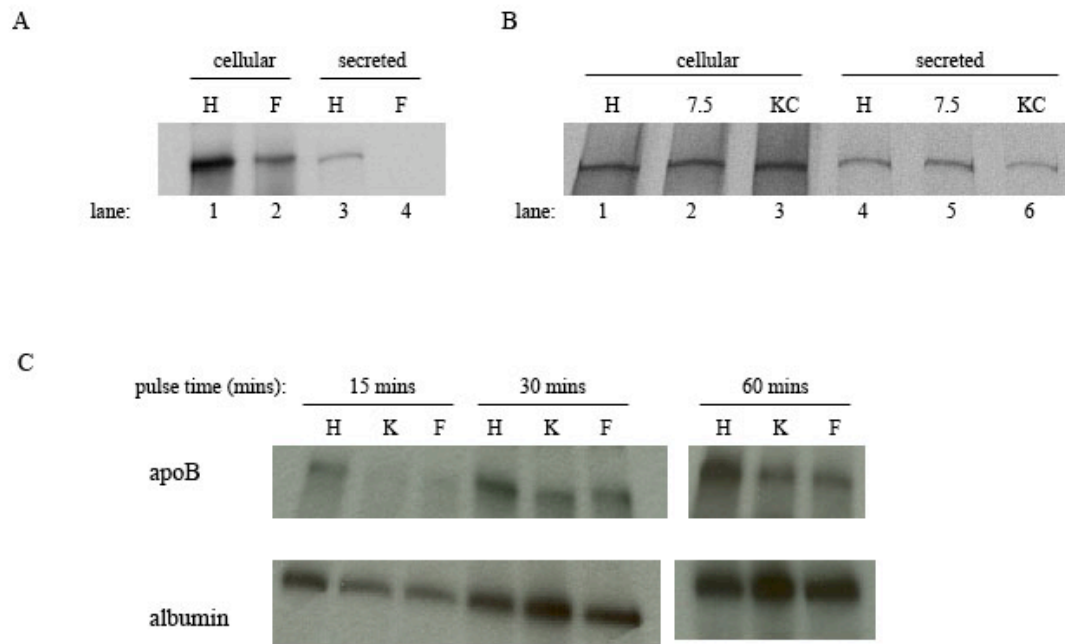


Fig. 1

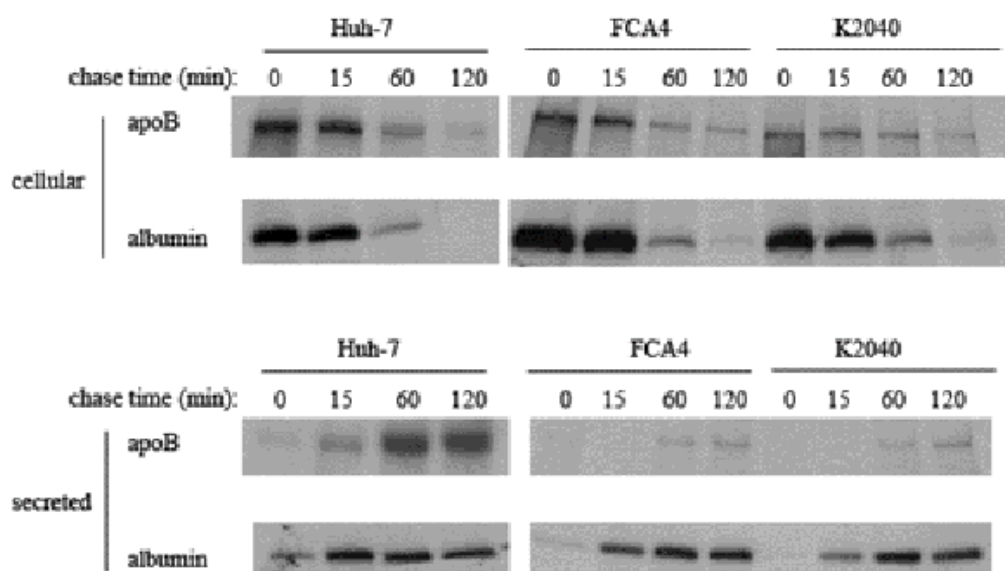


Fig. 2

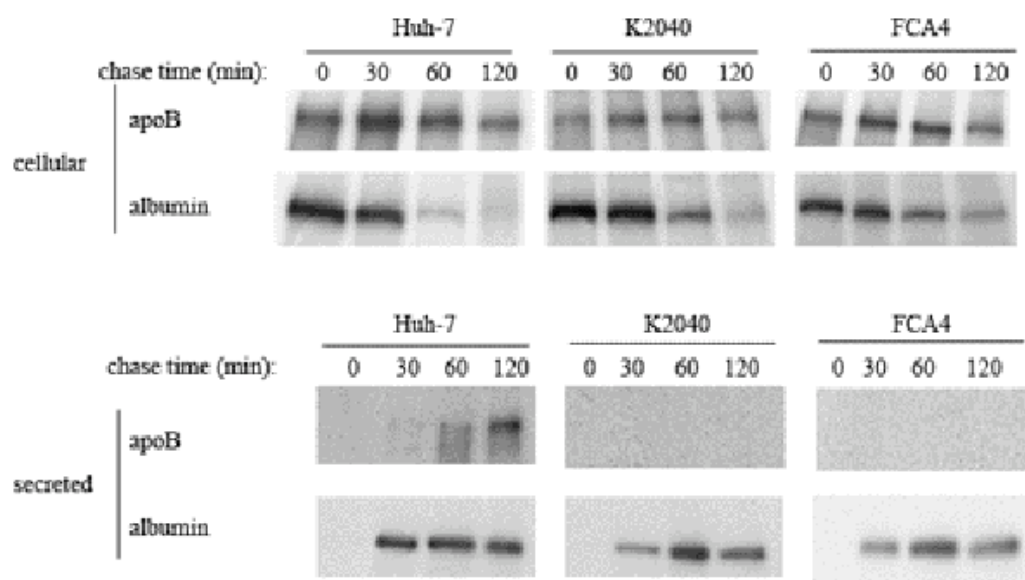


Fig. 3

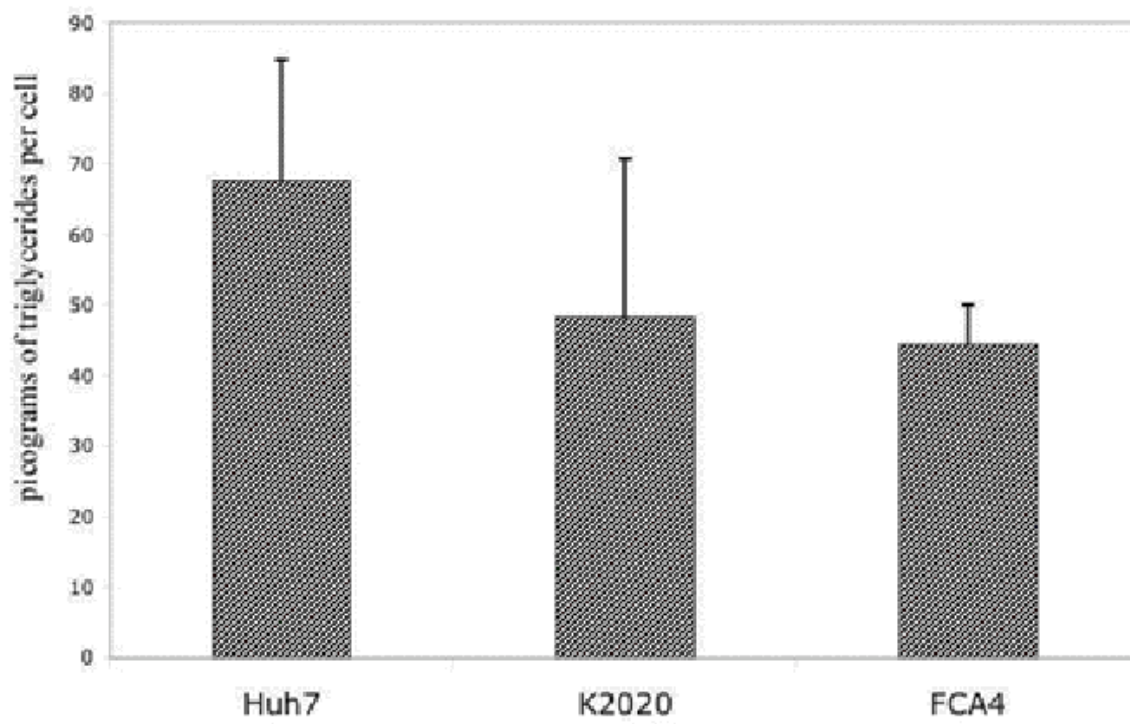


Fig. 4

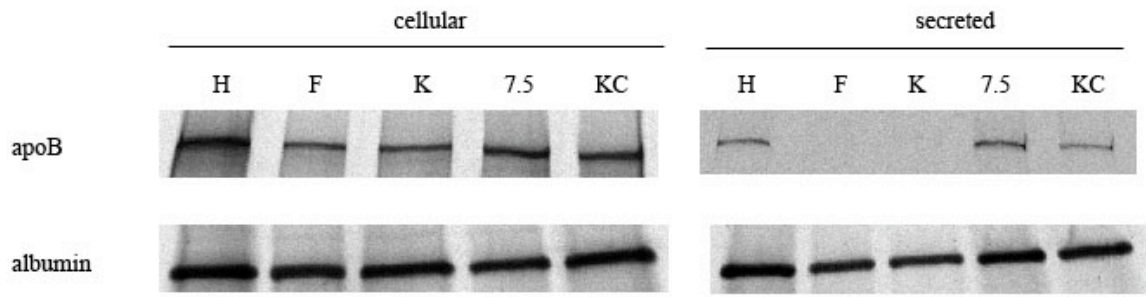


Fig. 5

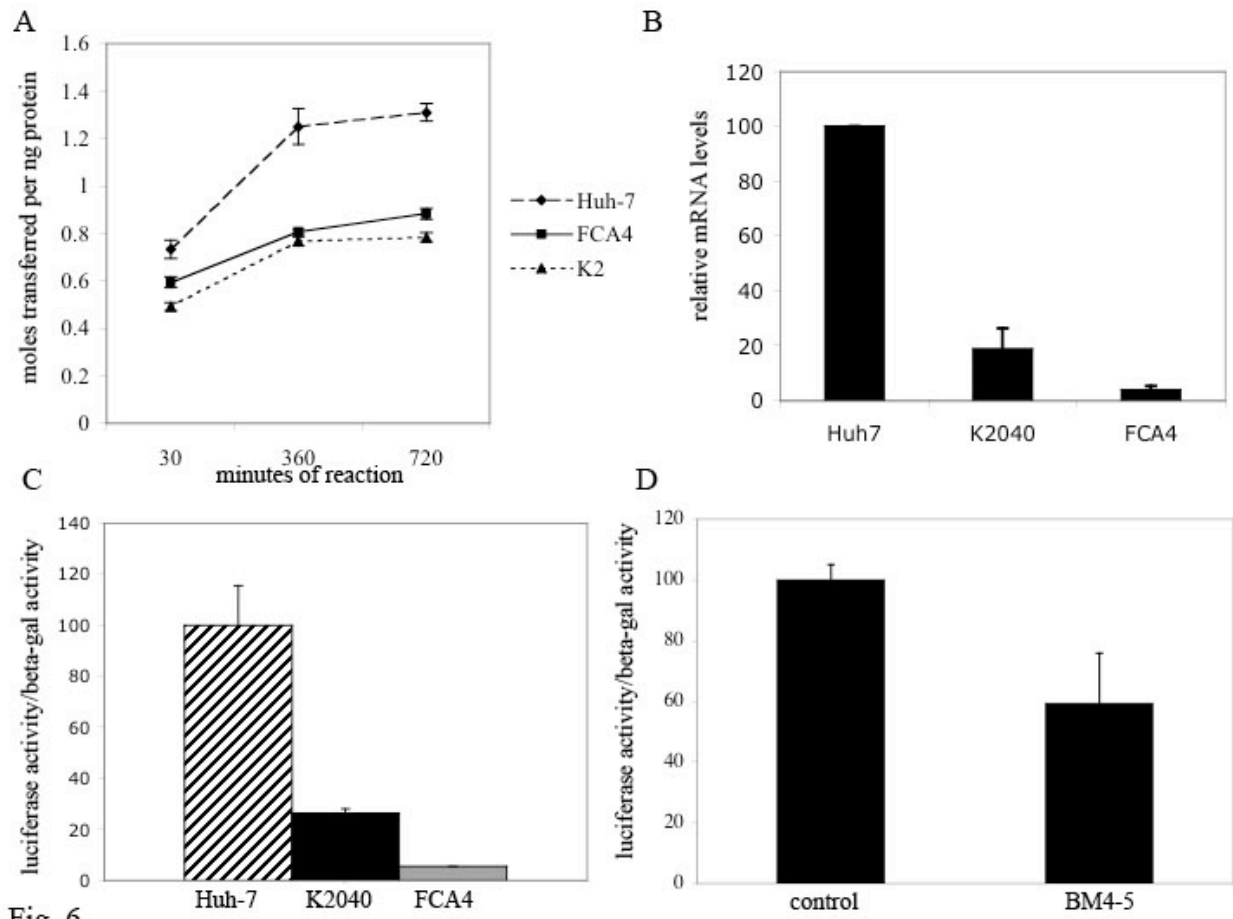
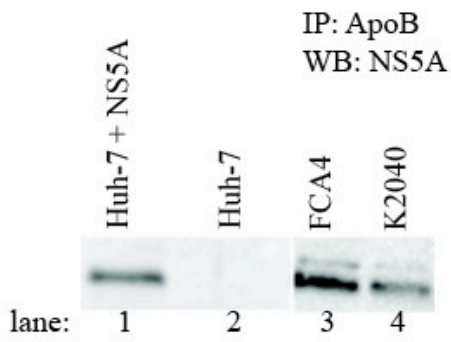


Fig. 6

A



B

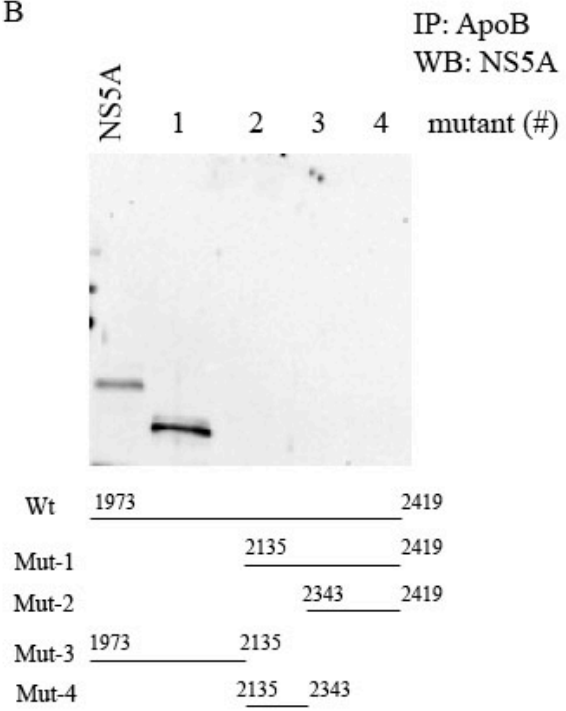


Fig. 7