The influence of cholesterol and lipid metabolism on host cell structure and hepatitis C virus replication

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Abstract: The hepatitis C virus (HCV) replicates on a membrane protein complex composed of viral proteins, replicating RNA, and altered cellular membranes. Small-molecule inhibitors of cellular lipid–cholesterol metabolism such as 25-hydroxycholesterol, cerulenin, lovastatin, and GGTI-286 all show a negative effect on HCV replication. Perturbation of host cell lipid and cholesterol metabolism can disrupt replication complexes by altering membranous structures where replication occurs. Changes in cholesterol and (or) lipid composition can have a general effect on membrane structure. Alternatively, metabolic changes can exert a more subtle influence over replication complexes by altering localization of host proteins through alterations in lipid anchoring. Here, we use Huh-7 cells harboring subgenomic HCV replicons to demonstrate that 25-hydroxycholesterol, cerulenin, lovastatin, and GGTI-286 do not disrupt the membranous web where replication occurs, whereas cholesterol-depleting agents such as β-cyclodextrin do. Cellular imaging suggests that the HCV RNA can remain associated with subcellular compartments connected with replication complexes in the presence of metabolic inhibitors. Therefore, at least 2 different molecular mechanisms are possible for the inhibition of HCV replication through the modulation of cellular lipid and cholesterol metabolism.

Key words: hepatitis C virus, lipid metabolism, fluorescence microscopy, electron microscopy, membranous web, statins.

Résumé : Le virus de l'hépatite C (HCV) se réplique au sein d'un complexe membranaire de protéines, composé de protéines virales, d'ARN en réplication et de membranes cellulaires altérées. De petites molécules inhibitrices du métabolisme cellulaire du cholestérol et des lipides comme le 25-hydroxycholestérol, la cérulénine, la lovastatine et le GGTI-286 démontrent toutes un effet négatif sur la réplication du HCV. Le perturbation du métabolisme du cholestérol et des lipides de la cellule hôte peut désorganiser les complexes de réplication en altérant les structures membranaires lorsque la réplication survient. Les changements dans la composition en cholestérol ou en lipides peut avoir un effet général sur la structure de la membrane. Alternativement, des changements métaboliques peuvent exercer des influences plus subtiles sur les complexes de réplication en altérant la localisation des protéines de l'hôte à travers des altérations de l'ancrage lipidique. À l'aide des cellules Huh-7 qui comportent des réplicons subgénomiques du HCV, nous démontrons ici que le 25-hydroxycholestérol, la cérulénine, la lovastatine et le GGTI-286 ne désorganisent pas le tissu membranaire où se déroule la réplication, alors que des agents épuisant le cholestérol comme la β-cyclodextrine y parviennent. L'imagerie cellulaire suggère que l'ARN du HCV puisse rester associé aux compartiments cellulaires associés eux-mêmes aux complexes de réplication en présence d'inhibiteurs métaboliques. Ainsi, il existe au moins deux mécanismes moléculaires possibles causant l'inhibition de la réplication du HCV à travers la modulation du métabolisme des lipides et du cholestérol.

Mots clés : virus de l'hépatite C, métabolisme des lipides, microfluoroscopie, microscopie électronique, tissu membranaire, statines.

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Introduction

Positive-strand RNA viruses typically replicate their genomes in association with distinct cellular membrane alterations induced in host cells (Bolten et al. 1998; Gosert et al. 2000, 2002; Guo et al. 2004; Kushner et al. 2003; Pedersen et al. 1999; Schaad et al. 1997; Teterina et al. 2001). These altered cellular membranes may lead to pathology and cellular destruction. In hepatitis C virus (HCV) infection, it is generally thought that liver damage is primarily due to the host immune response to infection, but changes in cellular architecture may also directly contribute to liver cell damage and cell death (Darling and Wright 2004). Thus, the study of HCV-induced membrane alterations may elucidate the mechanisms of HCV-induced liver cell injury and allow the development of novel drugs for the treatment of HCV infection.

Previous studies have shown that expression of the HCV polyprotein induces distinct membrane alterations (Egger et al. 2002; Gosert et al. 2003; Moradpour et al. 2003). Expression of the HCV polyprotein induced 2 main membrane alterations: (*i*) a membranous web consisting of vesicles embedded in a membranous matrix of circular or tightly undulating membranes; and (*ii*) tightly associated vesicles that were often found surrounding the membranous web. Lipid droplets, known to occur as a consequence of HCV infection, were also frequently observed. The vesicles of the membranous web were ~85 nm in diameter. The second type of vesicles were irregular, and their membranes were in such close contact that 2 bilayers often fused to form a trilayer, resulting in contiguous vesicles (Egger et al. 2002; Gosert et al. 2003; Moradpour et al. 2003).

The nonstructural protein NS3-4A complex was found to induce large amounts of smooth single vesicles, whereas the NS4B protein gave rise to the membranous web while at the same time reducing the amount of rough endoplasmic reticulum (rER). The rER that remained was often found associated, if not in continuity, with the membranous web. This observation, as well as the notion that NS4B colocalizes with the rER marker disulfide isomerase (Hugle et al. 2001), suggests that the membranous web is rER-derived. Immunocytochemical preparations of cells expressing the entire HCV polyprotein were analyzed to determine the localization of the HCV proteins. The membranous web was labeled with antibodies recognizing the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B and the structural proteins core E1 and E2 (Egger et al. 2002). All HCV nonstructural proteins were found to be localized to the membranous web and associated lipid droplets in cells overexpressing the HCV open reading frame (ORF), and in Huh-7 cells harboring subgenomic HCV replicons (Egger et al. 2002; Gosert et al. 2003; Moradpour et al. 2003). No label was observed on other membrane features, with 2 exceptions: the rER, which was sometimes weakly labeled with structural proteins, and the lipid droplets, which were intensely labeled with anticore antibody. The contiguous vesicles were not labeled. Therefore, all HCV proteins tested were found to be associated with 1 or more of the cellular alterations.

This is consistent with in vivo data from HCV-infected chimpanzees (Pfeifer et al. 1980). Investigators observed web-like structures in 6 of 7 chimpanzees infected with non-A, non-B hepatitis from the serum components of HCV-infected patients. These were described as "sponge-like inclusions". This indicates that the membranous web observed in vitro can also occur in vivo. Gene expression profiling of infected chimpanzees has also confirmed a direct linkage with lipid metabolism (Su et al. 2002) that might contribute to the alterations of membranous structures or altered protein lipidation (Ye et al. 2003).

Electron microscopy and in situ hybridization has revealed that the majority of HCV-specific, positive-strand RNA is associated with the membranous web (Gosert et al. 2003). This has led to the conclusion that the membranous web contains both the viral nonstructural proteins as well as replicating viral RNA. Moradpour et al. (2003) used metabolic labeling with bromouridine-triphosphate to reveal that the membranous web is indeed the site of viral RNA synthesis and, therefore, represents the replication complex of HCV. Ivashkina et al. (2002) showed that RNA replication takes place in a compartment that sustains endoglycosidase H - sensitive glycosylation; this implicates the ER-derived membranous web as the site of localization of the HCV replication complex and subsequently of HCV RNA synthesis.

Shi et al. (2003) showed that replicating HCV RNA and the nonstructural proteins colocalize on cytoplasmic membrane structures derived from the ER and Golgi apparatus. These structures that are detergent-resistant and cofractionate with caveolin-2. These results indicate that the HCV replication complex may form on lipid-raft-associated membrane complexes (Shi et al. 2003).

Core protein, expressed exclusively in the liver of transgenic mice, causes the development of steatosis (Moriya et al. 1997) and thereafter hepatocellular carcinoma (Moriya et al. 1998). Hope and McLauchlan (2000) showed lipid droplet and ER membrane localization of the core protein. In lipid-deficient media, where lipid droplets are not formed, core protein was found in a reticular cytoplasmic pattern, indicating that it is associated with the ER membrane. Although the processes involved in their formation are not yet clear, lipid droplets are thought to bud from ER membranes (Murphy and Vance 1999). Thus, the core may bind to a common lipid or protein component present in both the lipid droplets and the ER membrane.

Not only is NS5A localized to the ER and Golgi apparatus but it also colocalizes with the core protein in lipid droplets. Shi et al. (2002) showed that NS5A interacts with ApoA1, a lipid-binding protein and 1 of the major protein components of high-density lipoprotein particles involved in serum cholesterol regulation. NS5A is capable of associating with a number of components of the cellular lipid metabolic pathways and may thus contribute to HCV-induced steatosis. NS5A was not able to induce lipid droplet formation alone but was localized to core-induced lipid droplets. The NS5A protein may change the contents, or affect the stability, of the lipid droplets (Shi et al. 2002).

Small molecules that disrupt HCV replication may do so by directly inhibiting nonstructural proteins such as the NS3 protease or the NS5B RNA-dependent RNA polymerase, or by affecting the local environment that is required for replication to occur. Since HCV replicates on distinct membranous web structures in multiprotein complexes, the latter mechanism for antiviral activity may come about through

the inhibition of protein–protein and (or) protein–lipid interactions or by disruption of the cellular structures in which replication occurs. Examples of small molecules that inhibit HCV replication, through mechanisms other than direct inhibition of its nonstructural proteins, include 25-hydroxycholesterol and cerulenin, both of which decrease fatty acid biosynthesis by affecting the mevalonate pathway, as well as inhibiting HCV replication in a dose-dependent manner at nontoxic concentrations (Su et al. 2002). This implicates a direct role of cellular lipid metabolism in the HCV life cycle and suggests that insight into that life cycle may lead to the development of novel antiviral therapeutics (Su et al. 2002). Lovastatin, a potent inhibitor of 3-hydroxy-3-methylgluatryl coenzyme A (HMG-CoA) reductase that catalyzes the conversion of HMG-CoA to mevalonic acid (the rate-limiting step in the synthesis of cholesterol) (Grimbert et al. 1994), and GGTI-286, an inhibitor of geranylgeranylation, also inhibit HCV replication by an indirect route (Ye et al. 2003).

In this study we investigated the influence of these small molecules on the host cell structure using a combination of fluorescence and electron microscopy techniques. We establish that while the disruption of the host-cell lipid structures does inhibit HCV replication in Huh-7 cells bearing subgenomic HCV replicons, the small-molecule inhibitors studied do not function in this way. Rather, a more specific mechanism involving the perturbation of the localization–function of the replication complex is implicated.

Materials and methods

Cell culture

Cell monolayers of the human hepatoma cell line Huh-7 were grown in Dulbecco's modified minimal essential medium (DMEM) (Invitrogen Canada, Burlington, Ont.) supplemented with 100 nmol nonessential amino acids/L, 50 U penicillin/mL, 50 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (CanSera International, Rexdale, Ont.) or 10% charcoal–dextran treated, membrane-filtered FBS (Gemini Bio-Products, Woodland, Calif.). G418 was added at a concentration of 250 μg/mL to the Huh-7 cells stably expressing HCV replicons. The pFK-I389neo/NS3-3′/5.1 and pFK-I389luc/NS3-3′/5.1 plasmids, which contain HCV subgenomic replicons, were obtained from Ralph Bartenschlager (Institute of Hygiene, University of Heidelberg, Heidelberg, Germany). The replicons harbor either neo^R (pFK-I389neo/NS3-3′/5.1) or firefly luciferase (pFK-I389luc/NS3-3′/5.1) gene at the 5′ end but otherwise are identical and express HCV nonstructural proteins (NS3 to NS5B) from the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (Fig. 1A).

To create tricistronic HCV subgenomic replicons that harbor both the selection marker (neo^R) and reporter gene (firefly luciferase), pFK-I389luc/NS3-3′/5.1 was modified by inserting a SnaB I restriction site (oligonucleotides Neoluc1: CGATACGTAGG and Neoluc2: CGCGCCTACGTATCG) between the Nru I and Asc I restriction sites of pFK-I389luc/NS3-3′/5.1 to give the pLS1 plasmid. This cloning step simultaneously removed ~120 bp from the 3′ end of HCV IRES located before the luciferase gene. In the next step, pLS2 was constructed by inserting the BstU I Sma I fragment from pIRES (Clontech, Palo Alto, Calif.), which harbors EMCV IRES, into the SnaB I restriction site of pLS1. In the last step the Nru I Spe I fragment from pLS2 was used to replace the Pme I Spe I fragment of pFK-I389neo/NS3-3′/5.1. The resulting plasmid, pFK-I389neo/luc/NS3-3′/5.1, harbors 3 cistrons: neo^R, firefly luciferase, and the HCV nonstructural ORF, translated from HCV IRES, EMCV IRES, and EMCV IRES, respectively (Fig. 1A). All plasmid constructs were confirmed by sequencing.

In vitro transcription

In vitro transcripts were generated using MEGAscript™ (Ambion Inc., Austin, Tex.) according to the manufacturer's protocol. In brief, the template DNA was linearized with the restriction enzyme Sca I (New England BioLabs, Pickering, Ont.), precipitated for less than 30 min, and resuspended in RNase-free water to a concentration of 0.5 μg/μL. The in vitro reaction was set up and incubated at 37 °C for 2 h. To degrade the DNA template, 1 μL of DNaseI was added and incubated for another 15 min at 37 °C. The in vitro transcripts were then cleaned using the MEGAclear™ kit from Ambion Inc. according to the manufacturer's protocol. A 20 μL reaction usually produces 100 μg of RNA. The concentration was determined by measurement of the absorbance at 260 nm with an ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Del.), and RNA integrity was verified by electrophoresis using the Agilent 2100 bioanalyzer with the RNALabChip® kit according to the manufacturer's protocol.

Creating stable cell lines harboring subgenomic replicons

For electroporation, Huh-7 cells were washed twice with ice-cold PBS buffer and then resuspended in Cytomix transfection buffer at 10^7 cells/mL. RNA (10 μg) was mixed with 500 μL of cell suspension in the electroporation cuvette (gap width = 0.4 cm, Gene Pulser Xcell; Bio-Rad Laboratories, Hercules, Calif.) and immediately subjected to 1 pulse at 270 V, 960 μF. After 10 min of incubation at room temperature, the electroporated cells were diluted into 9 mL of growth medium and transferred into 100-mm plates. For selection of neo^R-expressing cells, the medium was replaced with fresh medium containing 500 μg of G418/mL after 24 h of incubation. Alternatively, RNA transfections were performed using DMRIE-C transfection reagent (Invitrogen, Carlsbad, Calif.) at the ratio of 5 μg of transfection reagent to 1 μg of RNA. Cultures were exposed to the RNA–lipid mixture for 6 h at 37 °C. Culture medium was supplemented with 500 μg of G418/mL 24 h after transfection.

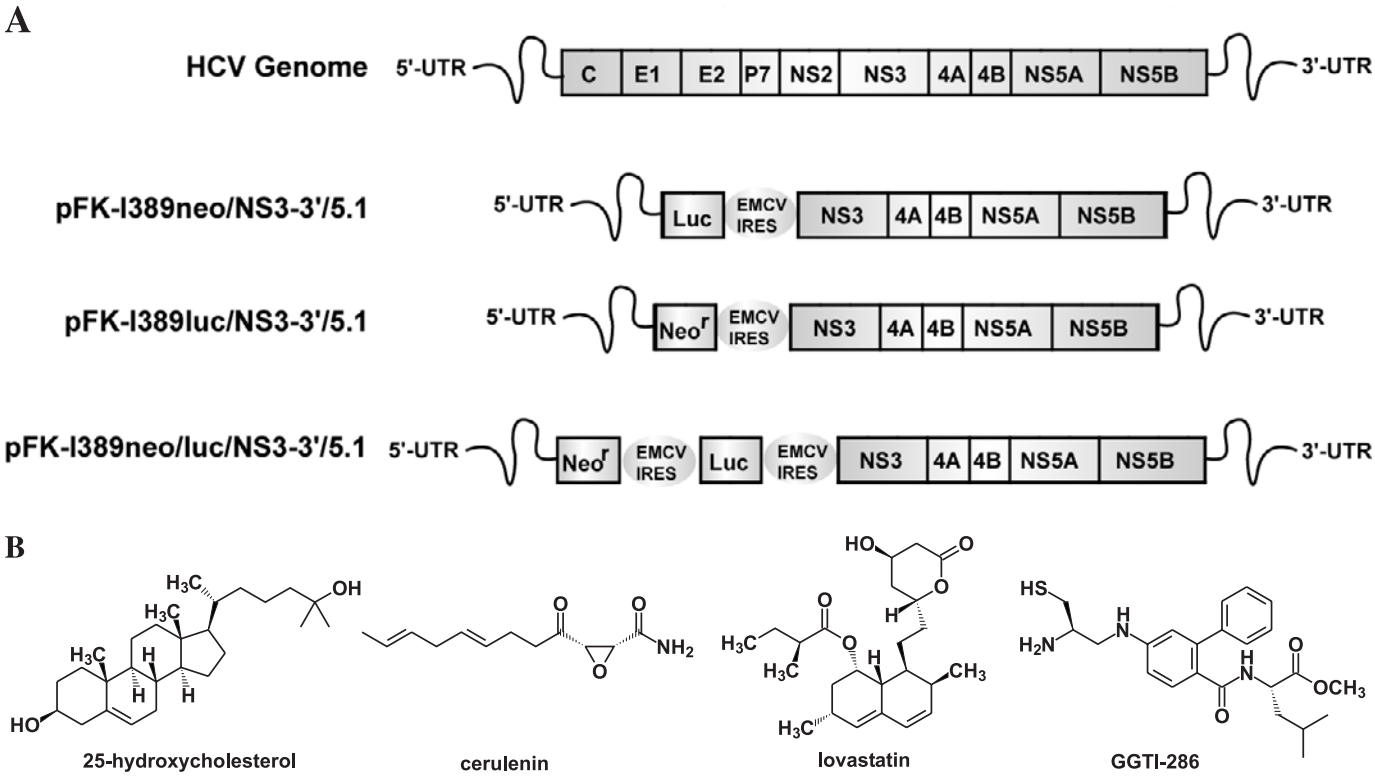
5′-end labeling of RNA with fluorescein

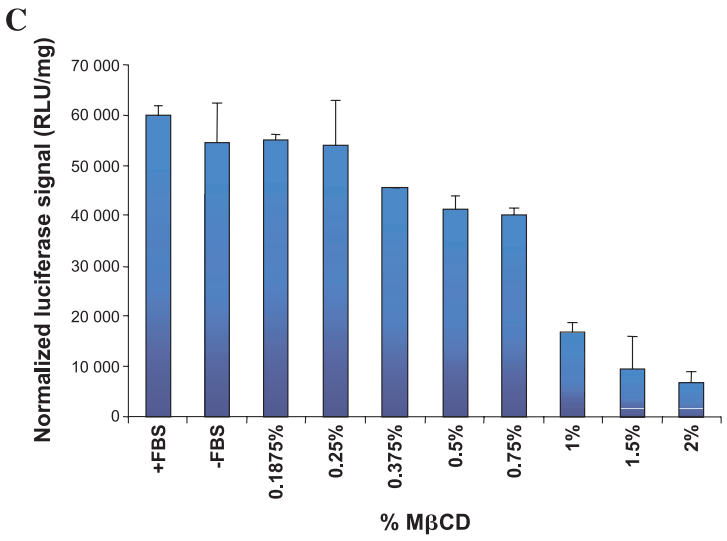
RNA in vitro transcripts were labeled with a fluorescein-maleimide label with the 5′-EndTag™ Nucleic Acid Labeling System from Vector Laboratories (Burlingame, Calif.). Briefly, 16 μg of HCV RNA, corresponding to 0.006 nmol of ends, was labeled with the thiol-reactive label fluorescein-maleimide. A thiophosphate is transferred from ATPγS to the 5′-hydroxyl group of the RNA by T4 polynucleotide kinase. After addition of the thiol functional group, a thiol-reactive label was chemically coupled to the 5′ end of the RNA.

Transient transfection of RNA in Huh-7 cells

Huh-7 cells were seeded at 5×10^4 cells/well in a 24-well plate to obtain cells at 70% to 80% confluency the next day.

Fig. 1. Comparison of different HCV subgenomic constructs. (A) The 3 different replicon variants used in this study. All 3 replicons encode the 3′ part of HCV polyprotein starting with NS3, which is translated from EMCV IRES. Two bicistronic variants, pFK-I389neo and pFKI389luc, encode at the 5′-end neomycin resistance and firefly luciferase genes, respectively, translated from HCV IRES and amino-terminally fused to the 16th amino acid of HCV core protein. Tricistronic variant pFK-I389neo/luc was derived from pFK-I389neo by inserting firefly luciferase gene translated from EMCV IRES between 2 cistrons originally present. (B) Small molecules that perturb cholesterol–lipid metabolism and show anti-HCV activity in replicon systems. (C) Effect of cholesterol depletion molecule MβCD on HCV RNA replication in E9 cells (Huh-7 cells stably replicating HCV RNA). Values were normalized by the total protein content of each sample and are shown as mean ± SD.





On the second day, the cells were washed twice with 1× PBS, pH 7.4, (Gibco–Invitrogen, Burlington, Ont.) and once with serum- and antibiotic-free DMEM (Gibco–Invitrogen). Then, the transfection complexes were prepared for each well as follows: 0.5 µg of the RNA of interest and 3 µL of DMRIE-C (Gibco–Invitrogen) were mixed in 500 µL of serum- and antibiotic-free DMEM. The lipid–RNA complexes were immediately added to the washed cells and incubated

for 2 h before 500 μL of DMEM solution with 20% FBS (or charcoal–dextran treated, membrane-filtered FBS) was added to the cells.

Replicon treatments

Cerulenin, 25-hydroxycholesterol, lovastatin, and GGTI-286 (Fig. 1B) were bought from Calbiochem (San Diego, Calif.). Lovastatin required activation for use in cell culture. Briefly, it was converted from its inactive lactone prodrug form to its active dihydroxy open acid form by first dissolving 52 mg in 1.04 mL of 95% ethanol and then adding 813 μL of 1N NaOH. The resulting solution was neutralized with 1N HCl to a pH of 7.2 and brought to a volume of 13 mL with distilled water. The final concentration was 10 mmol/L. Huh-7 cells were transfected as mentioned previously. Either cerulenin, lovastatin, or GGTI-286 was added to the cells at different concentrations when the 20% FBS (or charcoal–dextran treated, membrane-filtered FBS) and DMEM solution was added, usually 2 h after transfection. The cells were cultured and assays were preformed at 24 h after transfection. Methyl-β-cyclodextrin (MβCD) (Sigma–Aldrich Canada, Oakville, Ont.) was prepared as a 5% solution in serum- and antibiotic-free DMEM. Huh-7 cells stably replicating the HCV RNA were cultured in the absence of serum for 24 h; at this time the MβCD was added to the cells at various concentrations for 1 h time periods. The cells were then prepared for the respective assays.

Cytotoxicity assay

The cytotoxicity of MβCD was measured by using the CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega Corporation (Madison, Wis.). Briefly, the cells, treated as described, were washed 3× with 1× PBS and lysed with 10% Triton-X in a final volume of 100 μL for 45 min in a humidified incubator at 37 °C, 5% CO_2. For the assay, 50 μL of lysate was transferred to a 96-well plate; 50 μL of the substrate mix was added to each well; plates were incubated at room temperature for 30 min; and then 50 μL of stop solution was added to each well. Finally, the absorbance was recorded at 492 nm and 650 nm. Experiments were done in duplicate. Values of each sample are shown as mean ± SD.

Luciferase reporter assay and total protein quantification

Huh-7 cells were transfected as described previously (Su et al. 2002). The cells were washed once with 500 μL of 1× PBS and were then lysed with 100 μL of 1× cell culture lysis buffer (Promega Corp.) for 10 min at room temperature. Forty microliters per condition was transferred to a 96-well plate; 50 μL of luciferase assay substrate was added per well, and the relative light units were measured after a 2 s delay by an Lmax luminometer (Molecular Devices Corp., Sunnyvale, Calif.) using the SOFTmax Pro software. The total protein content of the different samples was quantified to normalize the luciferase reporter assay data. Experiments were done in duplicate. Values were normalized by the total protein content of each sample and are shown as mean ± SD. Total protein content of whole-cell extract lysates was quantified using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Mississauga, Ont.), similar to the well-documented Lowry assay. BSA was used as a protein standard. Typically, 100 μL of the alkaline copper tartrate solution and 800 μL of the dilute Folin reagent were added to 20 μL of the whole cell extract. The absorbance was read using a BioPhotometer (Eppendorf, Mississauga, Ont.) at 595 nm after 15 min of incubation at room temperature.

Fluorescence microscopy

Huh-7 cells were washed once with 1× PBS, pH 7.4, and fixed with 3.7% formaldehyde for 30 min at 4 °C. Oil red O (0.5% in isopropanol) (Sigma–Aldrich Canada, Oakville, Ont.) was diluted with water (3:2), filtered through a 0.45 μm filter, and incubated with the fixed cells for 30 min at room temperature. Cells were washed with 1× PBS and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The DAPI stock solution (Molecular Probes, Eugene, Ore.) was diluted to 300 nmol/L in 1× PBS. Then 500 μL of the 300 nmol/L solution was added, and cells were incubated for 2 min at room temperature. The cells were washed 3× with 1× PBS, covered with 50% glycerol in PBS, and were then ready for imaging. Stained lipid droplets, nuclei, and fluorescently tagged HCV RNA were visualized by fluorescent microscopy using an Axiovert 200M inverted microscope from Zeiss with the Axiovision 3.1 software. The cells were photographed with an AxioCam connected to the inverted microscope.

Electron microscopy

Twenty-four hours after seeding the Huh-7 cells stably replicating HCV pFK-I389neo/luc/NS3-3′/5.1 RNA at 5×10^4 cells/well in a 24-well plate, the medium was removed and replaced by DMEM + 10% FBS containing 2 μmol 25-hydroxycholesterol/L or 50 μmol activated lovastatin/L. Lovastatin required activation for use in cell culture as described earlier in replicon treatments. The treatment was done for 24 h. Treatment with MβCD was accomplished by preparing it as a 5% solution in serum- and antibiotic-free DMEM. Huh-7 cells stably replicating the HCV pFK-I389neo/luc/NS3-3′/5.1 RNA were cultured in the absence of serum for 24 h, then 0.5% MβCD was added to the cells for 1 h. The cells were then fixed for electron microscopy. Cells were washed once with 1× PBS and then fixed for 30 min at 4 °C with 0.04% glutaraldehyde and 2% formaldehyde. Cells were post-fixed in 0.2% uranyl acetate, pH 6.9, for 15 min, then washed in double distilled H_2O 3×. Dehydration and embedding was done following the K4M protocol for Lowicryls (low-temperature embedding resins) described in the Technical Note of Canemco & Marivac (Lakefield, Que.), Cat. Nos. 057, 0571, 058, and 0581 (www.canemco.com). Immunoelectron microscopy was done by an indirect method with GAM-G20 (Cedarlane® Laboratories Limited, Hornby, Ont.).

Results

To determine the general effects of cholesterol depletion on the ability of HCV RNA to replicate as well as the effects on cellular morphology of Huh-7 cells harboring HCV replicons, we studied the dose–response of MβCD, a known cholesterol sequestering agent, on Huh-7 cells harboring replicating HCV replicon RNA. We observed that both Huh-7 cells transiently transfected with HCV replicon RNA and

cells stably replicating HCV RNA pFK-I389neo/luc/NS3-3′/5.1 showed a dose-dependent decrease in replication as a function of MβCD concentration. Figure 1C shows the dose–response for transient transfections of pFK-I389luc/NS3-3′/5.1 HCV replicon RNA. The activity of replicons was confirmed, and all showed interferon gamma (IFN-γ) sensitivity (ca. 95%, 100 U/mL IFN-γ) as determined by firefly luciferase measurement or by quantitative reverse transcriptase-PCR (data not shown). Then, the lactate dehydrogenase (LDH) levels were quantified using the CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega. LDH cytotoxicity assays indicated that higher concentrations (>1%) of MβCD appeared toxic to Huh-7 cells.

Next, we used fluorescence microscopy to determine the general effects of MβCD on Huh-7 cell morphology. Cells were fixed and stained with Oil red O and counterstained with DAPI. Both light and fluorescence microscopy images of Huh-7 cells treated with different doses of MβCD clearly showed a dispersion of lipid droplets within the cells and a noticeable contraction of the cytoplasm volume (Figs. 2A–2F).

Fluorescence microscopy of Huh-7 cells transfected with either pFK-I389luc/NS3-3′/5.1 or pFK-I389neo/luc/NS3-3′/5.1 HCV replicon RNA displayed punctuated or dotted patterns of HCV RNA near to, and sometimes associated with, lipid droplets within the host cells, as previously reported (Gosert et al. 2003). Previous studies of HCV replicon RNA localization used fluorescence in situ hybridization (Gosert et al. 2003), but in this study we used 5′-fluorescein - labeled HCV replicon RNA because it maintains the ability to translate and replicate the HCV genome. This allows for both fixed and live cell imaging of RNA localization. Microscopic imaging of Huh-7 cells transiently transfected with 5′-FITC labeled pFK-I389luc/NS3-3′/5.1 RNA that were subsequently treated with varying concentrations of MβCD, fixed and stained with Oil red O, and counterstained with DAPI clearly showed the dispersion of the HCV RNA from the dotted patterns typically observed for untreated Huh-7 HCV-replicon-bearing cells (Figs. 2G–2K).

Fluorescence microscopy was also used to determine whether a discernable change in cellular morphology occurs in the presence of cholesterol–lipid metabolism-altering small molecules. Concentrations of 25-hydroxycholesterol, cerulenin, lovastatin, and GGTI-286, for which HCV replicon activity was inhibited by at least 50%, were used for imaging in fluorescence microscopy experiments (Fig. 3A). Knockdown of genes involved in lipid synthesis was confirmed in gene microarray studies, which indicated approximately 50% knockdown of mevalonate pathway genes by the small-molecule inhibitors of cholesterol–lipid metabolism (unpublished data). Huh-7 cells were treated with the corresponding inhibitor, fixed, stained with Oil red O, and counterstained with DAPI. The samples were then imaged using fluorescence microscopy. It was observed that neither 25-hydroxycholesterol nor cerulenin had a discernible affect on Huh-7 morphology, whereas cells treated with lovastatin and high doses of GGTI-286 appeared condensed (Figs. 3B–3G). The lipids in the lovastatin- and GGTI-286-treated cells were concentrated in a smaller area and thus seemed higher in density. These alterations in cellular morphology were minor compared with the effects of MβCD, suggesting that each of the small molecules affects HCV replication by a mechanism that is more specific than simply lowering cholesterol concentrations and affecting lipid structures, such as lipid rafts.

Since FBS is rich in a variety of lipids, including fatty acids and cholesterol, and lipid homeostasis is a balance between exogenous lipids and their de novo synthesis, we carried out the small-molecule inhibitor experiments in the presence of nonlipidated (charcoal–dextran treated) FBS. Under these conditions, the small-molecule inhibitors had a lesser effect on HCV replication (data not shown). This is likely due to the direct link between stimulation of de novo lipid synthesis (elevated gene expression of lipid genes) and cell viability, making inhibition of these pathways much more difficult. Cerulenin, however, retained much of its activity in the presence of nonlipidated FBS despite the elevated level of de novo lipid synthesis. These observations suggest that de novo fatty acid synthesis is required for viral replication.

The effects of the small-molecule inhibitors of cholesterol–lipid metabolism on RNA localization, and thereby localization of the replication complexes, were accomplished using 5′-FITC-labeled pFK-I389luc/NS3-3′/5.1 HCV replicon RNA transiently tranfected into Huh-7 cells (Fig. 4). Each compound was added to cells harboring fluorescently labeled HCV replicon RNA, and microscopy was performed at different time intervals to identify effects on cellular morphology. In each case, we observed that the dotted punctate cellular localization was retained upon treatment with the compounds at concentrations and treatment durations that led to significantly reduced HCV replication. Again, at higher doses or at longer exposure times, both lovastatin and GGTI-286 caused the condensing of the cytoplasm of the Huh-7 cells, making the lipid droplets appear more dense than in untreated cells. The viral RNA also appeared to be more condensed in fewer and larger patches (Fig. 4).

Electron microscopy was performed on Huh-7 cells harboring stably replicating pFK-I389neo/luc/NS3-3′/5.1 HCV replicon RNA. These cells showed distinct morphological changes when compared with the parental Huh-7 cells, as has been described previously (Gosert et al. 2003). Membranous web structures were observed and appeared to be retained in cells treated with 25-hydroxycholesterol. Treatment was done in parallel on cells that were subsequently evaluated for HCV replication levels by luciferase assay and by quantitative RT-PCR, both of which confirmed a greater than 75% decrease in replication. Under these conditions the membranous web structures remained intact according to electron microscopy experiments (Fig. 5). These observations are consistent with the fluorescence microscopy experiments, which suggests that the mechanism for inhibition of HCV replication by 25-hydroxycholesterol is not simply depletion of cellular cholesterol levels and subsequent disruption of the general membrane structures associated with the HCV replication complex. Rather, the mechanism must involve a more subtle alteration of a protein–protein, protein–RNA, and (or) protein–lipid interaction. This would be distinct from the disruption caused by MβCD, which likely inhibits HCV replication by disrupting lipid raft structures where replication is thought to occur (Shi et al. 2003). It is well known that MβCD can disrupt lipid raft structures by altering their cholesterol levels. The clear morphological difference in fluorescence microscopy experiments establishes

Fig. 2. Effect of cholesterol depletion molecule MβCD on lipid content of E9 cells. Panels A–F show both light (upper) and fluorescence (lower) microscopy images of replicon-bearing Huh-7 (pFK-I389neo/luc/NS3-3′/5.1) cells treated with MβCD, fixed and stained with Oil red O, and counterstained with DAPI. The cells were cultured in the absence of serum for 24 h and then treated with MβCD at 0% (A), 0.25% (B), 0.5% (C), 1.0% (D), 1.5% (E), and 2.0% (F). Panels G–K show fluorescence microscopy images of Huh-7 cells transiently transfected with 5′-FITC labeled replicon RNA (pFK-I389luc/NS3-3′/5.1), treated with MβCD, fixed, stained with Oil red O, and counterstained with DAPI. The cells were cultured in the absence of serum for 24 h and then treated with MβCD at 0% (G), 0.25% (H), 0.5% (I), 1.0% (J), and 2.0% (K). For all images in this figure, bar = 30 μm.

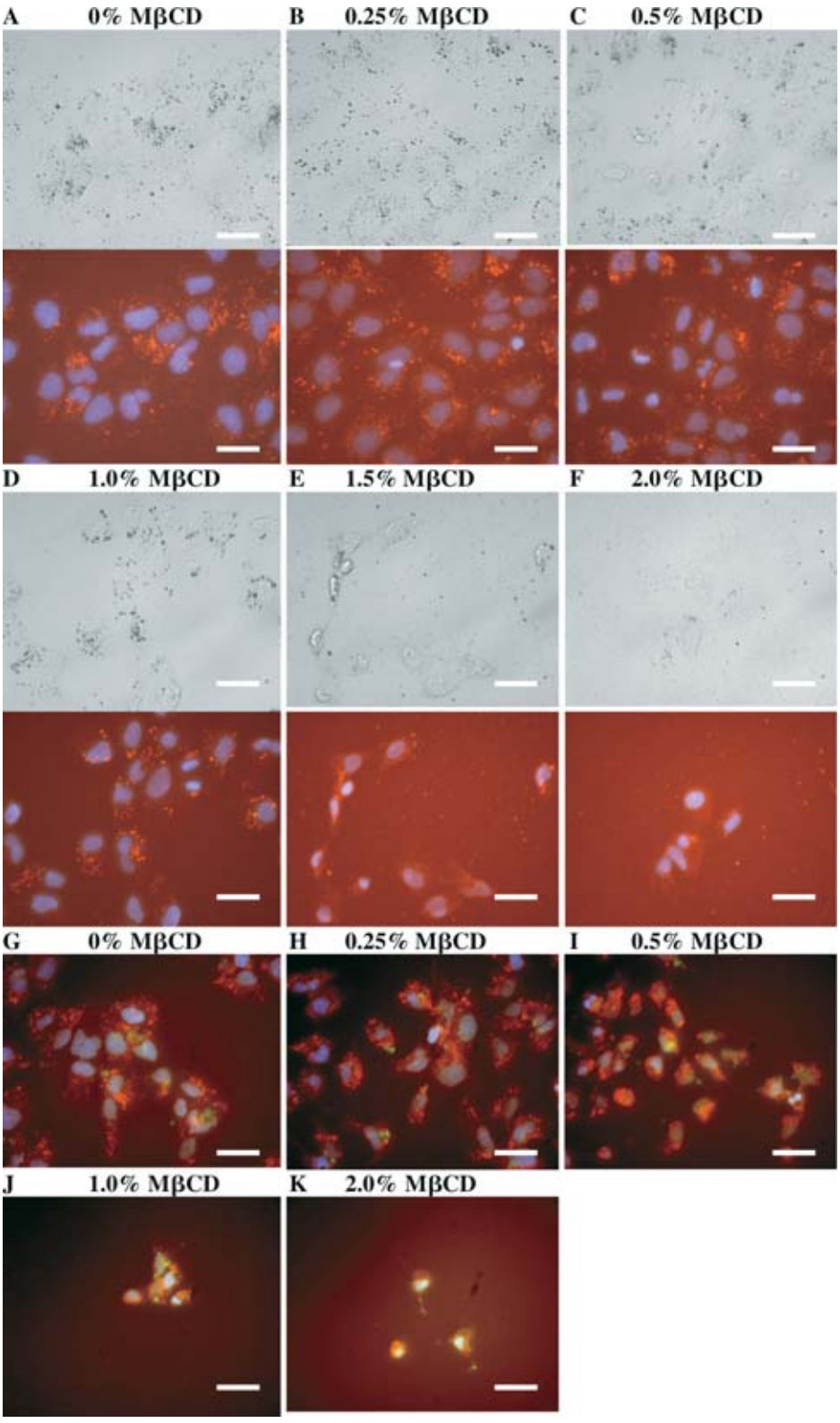
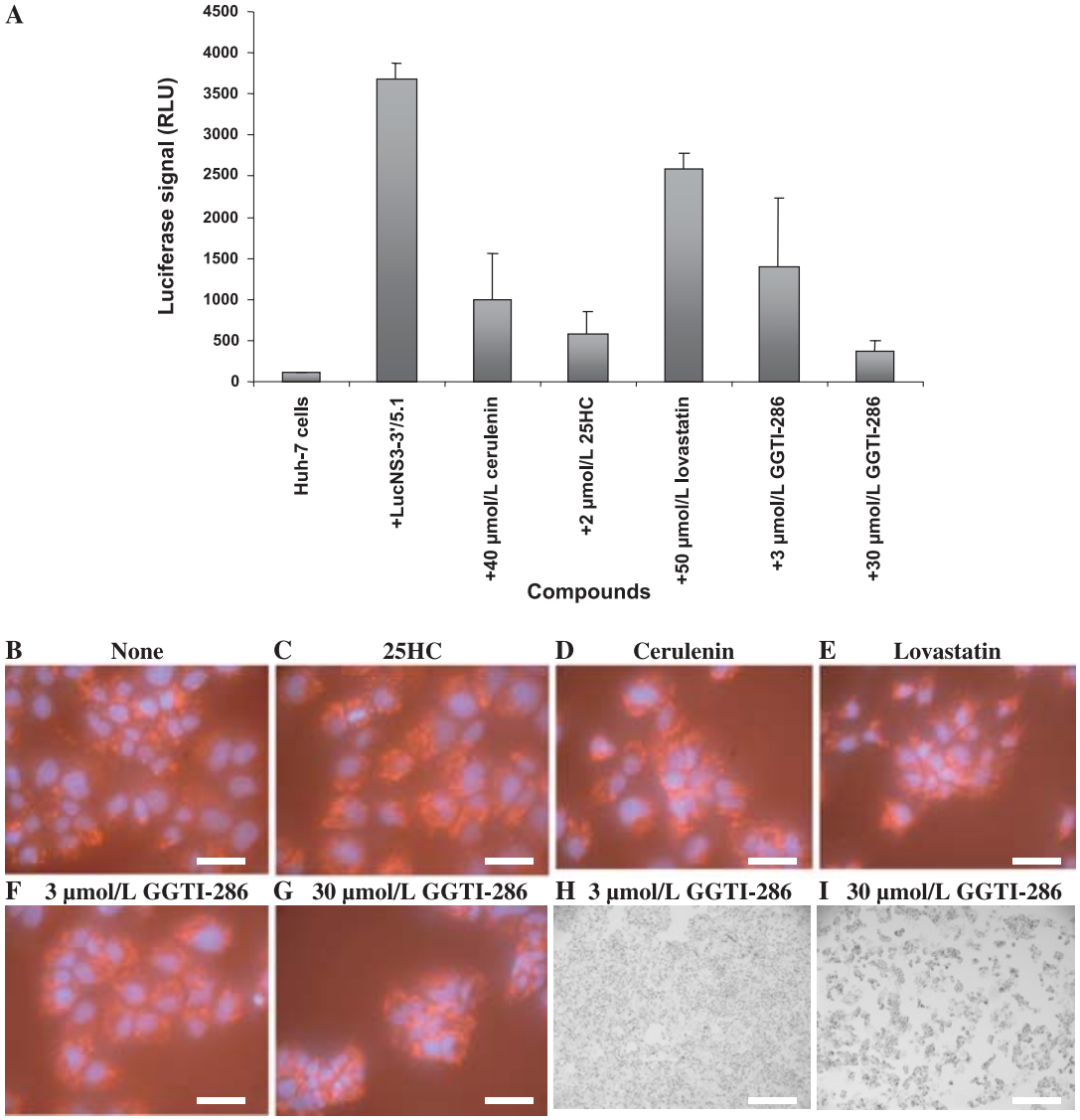


Fig. 3. (A) Effect of inhibitors of cholesterol–lipid biosynthesis on HCV RNA replication in Huh-7 cells transiently transfected with replicating pFK-I389/luc/NS3-3′/5.1 HCV RNA. Values are shown as mean ± SD. Panels B–H show the effects of inhibitors of cholesterol–lipid biosynthesis on lipid content and morphology. Cells were transfected with HCV replicon RNA, treated with inhibitors, fixed, stained with Oil red O, and counterstained with DAPI. Panels correspond to the following treatments: no treatment (B), 2 µmol 25-hydroxycholesterol/L (C), 40 µmol cerulenin/L (D), 50 µmol lovastatin/L (E), 3 µmol GGTI-286/L (F) as well as a light image of a different area of cells with the same treatment (H), and 30 µmol GGTI-286/L (G) as well as a light image of a different area of cells with the same treatment (I). For all images in this figure, bar = 30 µm.



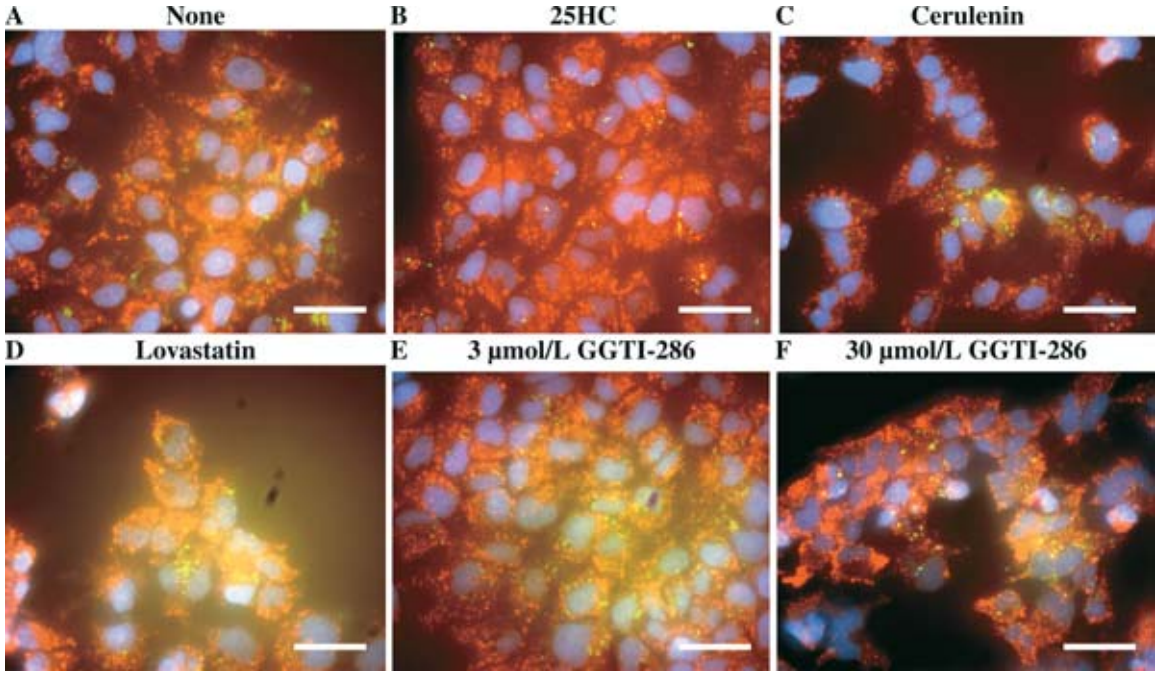
that this alteration causes the fluorescently labeled HCV RNA to become more diffuse and scattered throughout the cytoplasm.

Discussion

Because of the high abundance of both cholesterol and lipids within the cell, deregulation of pathways associated with cholesterol and lipid homeostasis can have both specific effects and general morphological effects on cellular structure. Depletion of cellular cholesterol is known to result in pleiotropic effects on cellular morphology and on membranous structures, including lipid rafts and caveolae structures (Razani et al. 2002), where multiprotein complexes implicated in cellular processes such as signaling are known to preferentially reside (Karlsson et al. 2004; Kenworthy et al. 2004). Perturbation of caveolae, either through cholesterol depletion or through caveolin protein misregulation, can arrest the function of multiprotein complexes that require the specific local environment to function. Colocalization of HCV proteins with caveolins suggests that HCV replication occurs on lipid rafts (Shi et al. 2003). HCV replication has also been shown to require hVAP-33, an integral vesicle membrane protein, which may be important for the localization of the HCV replication complex to lipid rafts (Gao et al. 2004).

The effects of cholesterol depletion are not restricted to mammalian cells and have even been demonstrated to influence the assembly and infectivity of viral particles such as

Fig. 4. Effect of inhibitors of cholesterol biosynthesis on localization of fluorescently labeled HCV RNA in Huh-7 cells transiently tranfected with 5′-end fluorescein-labeled HCV in-vitro translated RNA. Cells were transfected, treated with inhibitors, fixed, stained with Oil red O, and counterstained with DAPI. Panels correspond to the following treatments: no treatment (A), 2 μmol 25-hydroxycholesterol/L (B), 40 μmol cerulenin/L (C), 50 μmol lovastatin/L (D), 3 μmol GGTI-286/L (E), and 30 μmol GGTI-286/L (F). For all images in this figure, bar = 30 μm.



HIV-1 (Graham et al. 2003). Depletion of cellular cholesterol and lipids can influence the size and distribution of lipid droplets (made up of triglycerides, cholesterol esters, and membrane proteins surrounded by a phospholipid monolayer derived from the ER) (Londos et al. 1999; Sztalryd et al. 2003). Since HCV replication is thought to occur on membranous webs derived from the ER (Egger et al. 2002; Gosert et al. 2003; Moradpour et al. 2003) and HCV proteins are known to associate with and influence lipid droplets within hepatocytes (Hope and McLauchlan 2000; Shi et al. 2002, 2003), it is expected that the pleiotropic effects of cholesterol depletion should influence viral replication.

Depletion of cellular cholesterol using MβCD causes a decrease in HCV replication that is consistent with the general disruption of cellular membranes (Fig. 1). At concentrations of MβCD at which significant inhibition of HCV replication is observed, pleiotropic effects on the cellular morphology of the Huh-7 cells are clearly visible (Fig. 2). Furthermore, 5′-FITC-labeled subgenomic HCV replicon RNA is clearly dispersed and shows broad and diffuse staining patterns within MβCD-treated Huh-7 cells, which is quite different from the dotted punctate staining observed in the control cells (Fig. 2). These observations are consistent with the notion that HCV replication occurs in specific membranous environments (rafts) (Gao et al. 2004; Gosert et al. 2003; Moradpour et al. 2003), on membranous webs, and that cholesterol depletion is sufficient to disrupt these sites of replication.

Inhibitors of cholesterol biosynthesis can have a more complex influence on cellular processes than simply depleting cholesterol levels. The mevalonate pathway produces isoprenoids that are vital for diverse cellular functions, ranging from cholesterol synthesis to growth control (Goldstein and Brown 1990). The bulk-product of mevalonate metabolism, cholesterol, is obtained from 2 sources: (*i*) endogenously by synthesis from acetyl-CoA through mevalonate and (*ii*) exogenously from receptor-mediated uptake of plasma low-density lipoprotein (LDL). Mevalonate is also incorporated into nonsterol isoprenoids. Each cell in the body must balance the external and internal sources of cholesterol to sustain synthesis while avoiding overaccumulation of sterols. This balance is achieved through feedback regulation of at least 2 sequential enzymes in mevalonate synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase, as well as LDL receptor synthesis (Goldstein and Brown 1990).

One mechanism of regulation of the genes for the LDL receptor, HMG-CoA synthase, and HMG-CoA reductase is transcriptional regulation. The genes for each of these contain a consensus sequence (5′-CACC C/G C/T AC-3′) designated sterol regulatory element-1 (SRE-1). Sterol regulatory element binding proteins (SREBPs) bind to the SRE-1 sequence in these genes and help regulate their expression in the presence of sterols (Brown and Goldstein 1997). HMG-CoA reductase is also regulated on a post-transcriptional level by nonsterol regulation of mRNA translation, sterol and nonsterol degradation, and also by reversible inactivation of the enzyme by phosphorylation (Goldstein and Brown 1990).

Oxysterols, such as 25-hydroxycholesterol, are potent regulators of cell sterol levels, downregulating both the activity of HMG-CoA reductase as well as the number of LDL receptors on cells, and enhancing cellular cholesterol esterification by activation of acyl-CoA:cholesterol acyltransferase (Kilsdonk et al. 1995). Oxysterols can modulate cho-

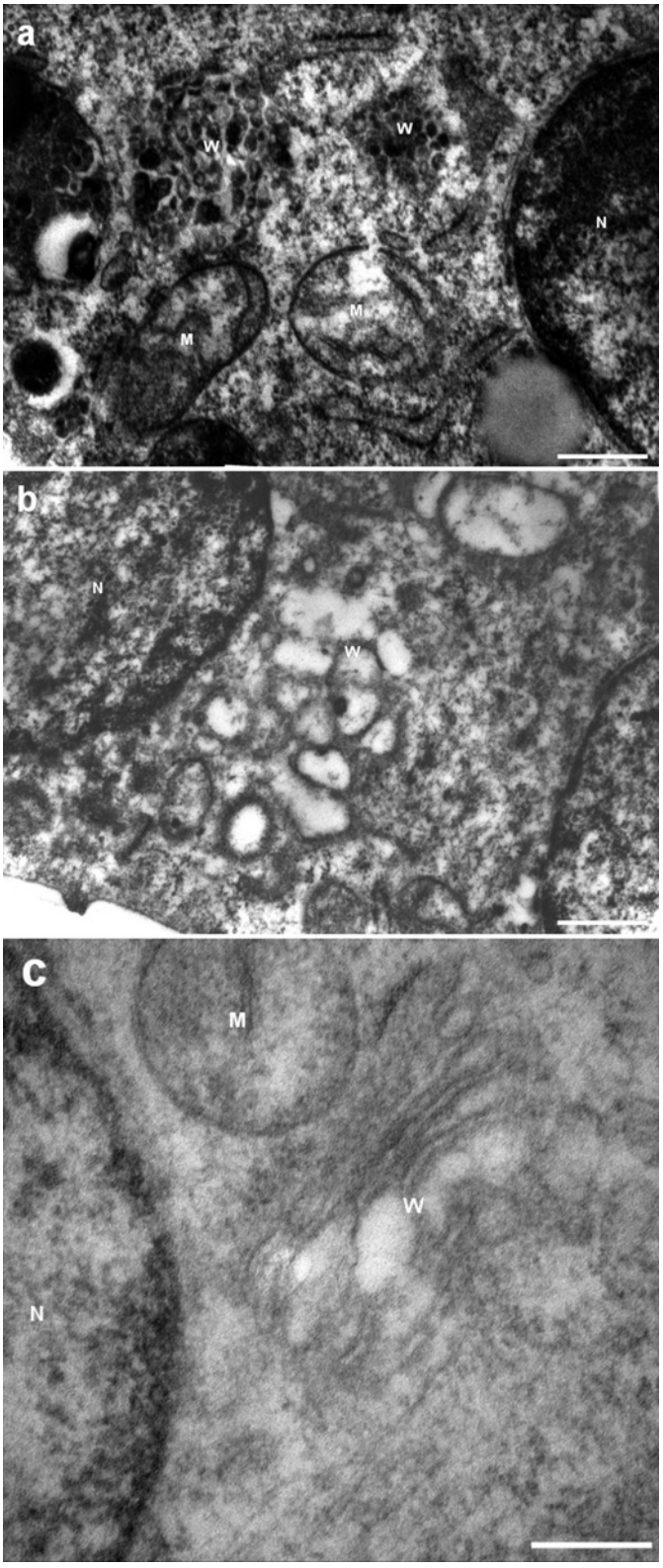


Fig. 5. Effect of 25-hydroxycholesterol treatment on the membranous web in Huh-7 cells stably replicating pFK-I389neo/luc/NS3-3′/5.1 HCV replicon RNA. Cells were cultured on coverslips in the presence of 2 μmol 25-hydroxycholesterol/L for 24 h. Cells were then fixed with fixation solution (0.1 mol Na_2HPO_4/L, 0.04% glutaraldehyde, 2% paraformaldehyde in PBS) for 2 h at 4 °C. Slides were imaged by electron microscopy. (*a*) Low-power image showing the membranous web. N, nucleus; W, membranous web. Bar = 0.4 μm. (*b*) High-power image of the membranous web. N, nucleus; M, mitochondria; W, membranous web. Bar = 0.2 μm. (*c*) High-power control image of Huh-7 cells stably replicating pFK-I389neo/luc/NS3-3′/5.1 HCV replicon RNA without 25-hydroxycholesterol treatment. N, nucleus; M, mitochondria; W, membranous web. Bar = 0.2 μm.

lesterol homeostasis at the level of cholesterol uptake and intracellular metabolism. 25-hydroxycholesterol has been found to be the most effective oxysterol at reducing cholesterol efflux (Kilsdonk et al. 1995). It is thought to inhibit SREBP-mediated gene transcription by inhibiting cleavage and translocation of the protein to the nucleus (Nohturfft et al. 2000), thus affecting both fatty acid and cholesterol biogenesis. 25-hydroxycholesterol has also been shown to inhibit HCV replication (Su et al. 2002).

Given the potent inhibitory activity of 25-hydroxycholesterol on cholesterol uptake and biogenesis, it would be expected that treatment of HCV replicon harboring Huh-7 cells would result in a similar phenotype as that of MβCD and that it would ultimately function to inhibit HCV replication by interfering with the lipid microdomain structure necessary for the host cell to support HCV replication. However, the phenotype observed when Huh-7 cells or Huh-7 cells harboring 5′FITC-labeled pFK-I389neo/luc/NS3-3′/5.1 HCV replicon RNA are treated with 25-hydroxycholesterol was quite different from that of MβCD under conditions in which replication was inhibited by more than 50%. Unlike MβCD, 25-hydroxycholesterol-treated Huh-7 cells retain normal morphology, and cells harboring fluorescently labeled replicon RNA retain a punctate dotted staining of the replicon RNA (Figs. 3 and 4), whereas in the MβCD-treated cells the staining is much more diffuse and is spread throughout the cytoplasm (Fig. 2). Electron microscopy of cells stably replicating pFK-I389neo/luc/NS3-3′/5.1 HCV replicon RNA with and without 25-hydroxycholesterol treatment confirm, on the nanometer scale, that features of the membranous web and lipid droplets are conserved upon treatment, implicating a mechanism that involves molecular interactions rather than disruption of nanometer-sized membranous features.

These results suggest that 25-hydroxycholesterol inhibits HCV replication by negatively regulating both cholesterol and fatty acid biosynthesis, and this effect may be synergistic. However, since the cholesterol depletion levels are clearly not equivalent to those for MβCD, it is likely that protein–protein or protein–lipid interactions necessary to form functional HCV replication complexes are inhibited by 25-hydroxycholesterol through its effects on isoprenoid and fatty acid synthesis.

Cerulenin, an antifungal antibiotic produced by *Cephalosporium caerulens*, has also been demonstrated to inhibit HCV replication in subgenomic replicon systems (Su et al. 2002). It has the structure (2R)(3S)-2,3-epoxy-4-oxo-7,10-*trans*,*trans*-dodecadienoylamide and is a potent inhibitor of fatty acid synthases of various organisms from microorganisms and plants to animals (Inokoshi et al. 1994). Cerulenin inhibits all types of fatty acid synthases, whether of the nonaggregated type (type II) or multifunctional enzyme system

(type I) (Inokoshi et al. 1994). Fluorescence microscopy experiments demonstrate that, at concentrations at which cerulenin inhibits HCV replication in Huh-7 cells harboring subgenomic replicons, no discernable morphological changes occur in the host cells, and viral RNA appears to maintain a cellular localization pattern similar to that observed in untreated cells. This, once again, implies a mechanism of HCV inhibition that is likely to be on the scale of molecular interactions.

Lovastatin is a potent inhibitor of HMG-CoA reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonic acid, the rate-limiting step in the synthesis of cholesterol (Grimbert et al. 1994). Lovastatin, a member of the family of substituted hexahydronaphthalene lactones, interferes with the biosynthesis of mevalonic acid by inhibition of HMG-CoA reductase (Alberts et al. 1980). The active form is the hydroxyl acid (opened lactone) structure. Treatment with lovastatin of HCV-replicon-containing cells reduced HCV RNA levels to ~25% of untreated controls. As lovastatin is a HMG-CoA reductase inhibitor, this result suggests that HCV RNA replication requires 1 or more products derived from mevalonate, the product of the HMG-CoA reductase reaction. Two of the mevalonate-derived isoprenoids, farnesyl (15 carbons) and geranylgeranyl (20 carbons), are attached to membrane proteins through a cysteine thioether linkage (Goldstein and Brown 1990). This process, known as protein prenylation, targets proteins to cellular membranes where they regulate many cellular functions ranging from vesicle budding and fusion to growth (Ye et al. 2003). HCV RNA levels can be rescued by providing mevalonate in a dose-dependent manner, and this rescue can be enhanced by providing exogenous LDL (Ye et al. 2003).

To define the mevalonate-derived products required for HCV RNA replication, Ye et al. (2003) cultured cells with or without lovastatin in the absence or presence of various metabolites whose synthesis requires HMG-CoA reductase. The result seen with LDL indicates that the depletion of a nonsterol end product of the mevalonate pathway is responsible for the effect on HCV RNA replication. In order to identify the nonsterol product, lovastatin-treated cells were supplemented with mevalonate-derived isoprenoids, geranylgeraniol and farnesol. At high concentrations, attainable in tissue culture cells, lovastatin depletes mevalonate sufficiently to lower the cellular pools of farnesyl and geranylgeranyl pyrophosphates, which are the donors in the protein prenylation reactions (Ye et al. 2003). Exogenous geranylgeraniol was able to rescue HCV RNA levels from lovastatin-induced suppression whereas farnesol was not. This indicates that lovastatin depletes the cells of geranylgeranylated proteins, and the depletion of 1 or more cellular geranylgeranylated proteins is required for HCV RNA replication (Ye et al. 2003).

Geranylgeranylated proteins mediate interaction of membranes with cytoskeletal proteins (Zhang and Casey 1996). These interactions are likely to be important in the formation of the ER-associated HCV replication complex, which contains viral RNA as well as the viral nonstructural proteins. Treatment with lovastatin disrupted the staining pattern of NS5A that is normally consistent with ER localization (Ye et al. 2003). This was also seen with treatment with GGTI-286, an inhibitor of geranylgeranyl transferase I (Ye et al. 2003). This enzyme is responsible for the addition of a geranylgeranyl group to the cysteine of the carboxy terminal CAAX motif of geranylgeranylated proteins. Addition of this inhibitor also resulted in a dose-dependent decrease in HCV RNA levels consistent with the hypothesis that HCV RNA replication requires a geranylgeranylated protein(s). Recently, a putative geranylgeranylated protein required for HCV RNA replication was identified to be the cellular protein FBL2 (Wang et al. 2005).

Fluorescence microscopy images suggest that the small-molecule inhibitors that modulate lipid metabolism can affect HCV replication by inhibiting the formation of a nonsterol biosynthetic product that may be a geranylgeranylated host protein or by more general disruption of the membrane structures that support the replicase complex. Electron microscopy experiments suggest that the host–virus interactions that are disrupted by 25-hydroxycholesterol occur on a molecular level and not through morphological alterations of host-cell structure. These results also suggest that morphological screening can identify molecules that may disrupt HCV replication by altering host structure or by dispersing HCV RNA from replication complexes. However, morphological screening does not easily identify molecules that inhibit host–virus interactions unless they alter the subcellular localization of a component of the replication complex.

Conclusions

Hepatitis C virus replicates on a membrane protein complex composed of viral proteins, replicating RNA, and altered cellular membranes. The function of this complex can be affected by both disrupting the unique cellular membranes of the membranous web or by altering specific components of the membrane complex that are required for replication. We have demonstrated that small-molecule inhibitors of cellular lipid–cholesterol metabolism, such as 25-hydroxycholesterol, cerulenin, lovastatin, and GGTI-286, all show a negative effect on HCV replication by perturbing a nonsterol host cell product. Cholesterol depletion can also affect HCV replication but through a different mechanism, which results in the breaking up of the cellular structures needed to support the replication complex. In addition, we demonstrate that simple microscopic experiments can be used to identify cellular morphologies that are likely to be unfavorable for HCV replication. Such screening experiments are currently under way.

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References

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C. et al. 1980. Mevinolin – a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme-A reductase and a cholesterol-lowering agent. Proc. Natl Acad. Sci. U.S.A. **77**: 3957–3961.

Bolten, R., Egger, D., Gosert, R., Schaub, G., Landmann, L., and Bienz, K. 1998. Intracellular localization of poliovirus plus- and minus-strand RNA visualized by strand-specific fluorescent in situ hybridization. J. Virol. **72**: 8578–8585.

Brown, M.S., and Goldstein, J.L. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell, **89**: 331–340.

Darling, J.M., and Wright, T.L. 2004. Immune responses in hepatitis C: is virus or host the problem? Curr. Opin. Inf. Dis. **17**: 193–198.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., and Bienz, K. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virology, **76**: 5974–5984.

Gao, L., Aizaki, H., He, J.W., and Lai, M.M.C. 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J. Virol. **78**: 3480–3488.

Goldstein, J.L., and Brown, M.S. 1990. Regulation of the mevalonate pathway. Nature (London), **343**: 425–430.

Gosert, R., Egger, D., and Bienz, K. 2000. A cytopathic and a cell culture adapted hepatitis-A virus strain differ in cell killing but not in intracellular membrane rearrangements. Virology, **266**: 157–169.

Gosert, R., Kanjanahaluethai, A., Egger, D., Bienz, K., and Baker, S.C. 2002. RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. J. Virol. **76**: 3697–3708.

Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H.E., Bienz, K., and Moradpour, D. 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. **77**: 5487–5492.

Graham, D.R.M., Chertova, E., Hilburn, J.M., Arthur, L.O., and Hildreth, J.E.K. 2003. Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with beta-cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts. J. Virol. **77**: 8237–8248.

Grimbert, S., Pessayre, D., Degott, C., and Benhamou, J.P. 1994. Acute hepatitis induced by HMG-CoA reductase inhibitor, lovastatin. Dig. Dis. Sci. **39**: 2032–2033.

Guo, Y.X., Chan, S.W., and Kwang, J. 2004. Membrane association of greasy grouper nervous necrosis virus protein A and characterization of its mitochondrial localization targeting signal. J. Virol. **78**: 6498–6508.

Hope, R.G., and McLauchlan, J. 2000. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J. Gen. Virol. **81**: 1913–1925.

Hugle, T., Fehrmann, F., Bieck, E., Kohara, M., Krausslich, H.G., Rice, C.M. et al. 2001. Hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. Virology, **284**: 70–81.

Inokoshi, J., Tomoda, H., Hashimoto, H., Watanabe, A., Takeshima, H., and Omura, S. 1994. Cerulenin-resistant mutants of *Saccharomyces cerevisiae* with an altered fatty acid synthase gene. Mol. Gen. Gen. **244**: 90–96.

Ivashkina, N., Wolk, B., Lohmann, V., Bartenschlager, R., Blum, H.E., Penin, F., and Moradpour, D. 2002. The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. J. Virol. **76**: 13088–13093.

Karlsson, M., Thorn, H., Danielsson, A., Stenkula, K.G., Ost, A., Gustavsson, J. et al. 2004. Colocalization of insulin receptor and insulin receptor substrate-1 to caveolae in primary human adipocytes – cholesterol depletion blocks insulin signalling for metabolic and mitogenic control. Euro. J. Biochem. **271**: 2471–2479.

Kenworthy, A.K., Nichols, B.J., Remmert, C.L., Hendrix, G.M., Kumar, M., Zimmerberg, J., and Lippincott-Schwartz, J. 2004. Dynamics of putative raft-associated proteins at the cell surface. J. Cell Biol. **165**: 735–746.

Kilsdonk, E.P.C., Morel, D.W., Johnson, W.J., and Rothblat, G.H. 1995. Inhibition of cellular cholesterol efflux by 25-hydroxycholesterol. J. Lipid Res. **36**: 505–516.

Kushner, D.B., Lindenbach, B.D., Grdzelishvili, V.Z., Noueiry, A.O., Paul, S.M., and Ahlquist, P. 2003. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. Proc. Natl Acad. Sci. U.S.A. **100**: 15764–15769.

Londos, C., Brasaemle, D.L., Schultz, C.J., Segrest, J.P., and Kimmel, A.R. 1999. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. Sem. Cell Dev. Biol. **10**: 51–58.

Moradpour, D., Gosert, R., Egger, D., Penin, F., Blum, H.E., and Bienz, K. 2003. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. Antiviral Res. **60**: 103–109.

Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y. et al. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J. Gen. Virol. **78:** 1527–1531.

Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K. et al. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nature Med. **4**: 1065–1067.

Murphy, D.J., and Vance, J. 1999. Mechanisms of lipid body formation. Trends Biochem. Sci. **24**: 109–115.

Nohturfft, A., Yabe, D., Goldstein, J.L., Brown, M.S., and Espenshade, P.J. 2000. Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes. Cell, **102**: 315–323.

Pedersen, K.W., van der Meer, Y., Roos, N., and Snijder, E.J. 1999. Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. J. Virol. **73**: 2016–2026.

Pfeifer, U., Thomssen, R., Legler, K., Bottcher, U., Gerlich, W., Weinmann, E., and Klinge, O. 1980. Experimental non-a, non-B hepatitis: four types of cytoplasmic alteration in hepatocytes of infected chimpanzees. Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. **33**: 233–243.

Razani, B., Woodman, S.E., and Lisanti, M.P. 2002. Caveolae: from cell biology to animal physiology. Pharmacol. Rev. **54**: 431–467.

Schaad, M.C., Jensen, P.E., and Carrington, J.C. 1997. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J. **16**: 4049–4059.

Shi, S.T., Polyak, S.J., Tu, H., Taylor, D.R., Gretch, D.R., and Lai, M.M.C. 2002. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. Virology, **292**: 198–210.

Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., and Lai, M.M.C. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. **77**: 4160–4168.

Su, A.I., Pezacki, J.P., Wodicka, L., Brideau, A.D., Supekova, L., Thimme, R. et al. 2002. Genomic analysis of the host response to hepatitis C virus infection. Proc. Natl Acad. Sci. U.S.A. **99**: 15669–15674.

Sztalryd, C., Xu, G.H., Dorward, H., Tansey, J.T., Contreras, J.A., Kimmel, A.R., and Londos, C. 2003. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. J. Cell Biol. **161**: 1093–1103.

Teterina, N.L., Egger, D., Bienz, K., Brown, D.M., Semler, B.L., and Ehrenfeld, E. 2001. Requirements for assembly of poliovirus replication complexes and negative-strand RNA synthesis. J. Virol. **75**: 3841–3850.

Wang, C., Gale, M., Jr., Keller, B.C., Huang, H., Brown, M.S., Goldstein, J.L., and Ye, J. 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol Cell. **18**: 425–434.

Ye, J., Wang, C.F., Sumpter, R., Brown, M.S., Goldstein, J.L., and Gale, M. 2003. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc. Natl. Acad. Sci. U.S.A. **100**: 15865–15870.

Zhang, F.L., and Casey, P.J. 1996. Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. **65**: 241–269.