Domain 3 of Hepatitis C Virus Core Protein Is Sufficient for Intracellular Lipid Accumulation

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Background. Hepatitis C virus (HCV) is a major cause of liver disease worldwide, with steatosis, or “fatty liver,” being a frequent histologic finding. In previous work, we identified sequence polymorphisms within domain 3 (d3) of genotype 3 HCV core protein that correlated with steatosis and in vitro lipid accumulation. In this study, we investigated the sufficiency of d3 to promote lipid accumulation, the role of HCV genotype in d3 lipid accumulation, and the subcellular distribution of d3.

Methods. Stable cell lines expressing green fluorescent protein (GFP) fusions with isolates of HCV genotype 3 core steatosis-associated d3 (d3S), non–steatosis-associated d3 (d3NS), and genotype 1 d3 (d3G1) were analyzed by means of immunofluorescence, oil red O (ORO) staining, and triglyceride quantitation.

Results. Cells that expressed d3S had statistically significantly more ORO than did cells expressing d3NS or d3G1 (\(P < .001\), respectively), as well as higher triglyceride levels (\(P = .03\) and \(.003\), respectively). Immunofluorescence analysis showed that d3 does not colocalize to lipid droplets but partially colocalizes to the Golgi apparatus.

Conclusions. Our results suggest that HCV core d3 is sufficient to mediate the accumulation of lipid by means of a mechanism that is independent of domains 1 and 2. Our results also suggest that altered lipid trafficking may be involved.
associated with lipid accumulation in HCV genotype 3 infection and identified specific polymorphisms at amino acid residues 182 and 186 within domain 3 (d3) of the core protein that correlated with the presence or absence of steatosis in a small group of patients [28]. We recapitulated these differences in an in vitro system of hepatocyte lipid accumulation and thus demonstrated that core proteins that carry these polymorphisms induce more lipid accumulation.

We report here the results of follow-up studies we performed to test the hypothesis that d3 alone is sufficient to cause intracellular lipid accumulation. We demonstrate that HCV core d3 green fluorescent protein (GFP) fusions promote more lipid droplets of greater size and increased triglyceride content. We show that d3 sequence differences within genotype 3 and between genotypes 3 and 1 determine the amount of lipid accumulation and that d3 does not exert its action by direct interaction with lipid droplets but appears to partially colocalize with the Golgi apparatus.

METHODS

HCV constructs. The HCV core sequences that were used as templates for subsequent experiments included steatosis-associated d3 (d3S) (HCV1 DCRI, GenBank accession no. EU099414), non–steatosis-associated d3 (d3NS) (HCV11 DCRI, GenBank accession no. EU099415) [28], and genotype 1 d3 (d3G1) (HCV-N, GenBank accession no. AF139594, provided by Dr Steve Weinman, University of Texas Medical Branch, Galveston) [30].

Plasmid construction. The plasmid named “78” (provided by Dr Brian Doehle, Duke University Medical Center, Durham, NC) was used for generating GFP fusions. Plasmid 78 contained the backbone of pcDNA3 ligated with an NdeI-EcoRI fragment from pEGFP-C, which contained part of the cytomegalovirus promoter, the GFP open reading frame, and part of the multiple cloning site. We designed custom oligonucleotides (Table 1) to generate the constructs d3S, d3NS, d3G1, and domain 2 (d2) retained with d3 (d2-d3). We used EcoRI and XbaI, which we subsequently cloned into digested and purified vector. Ligated products were transformed into Escherichia coli, and colonies were selected after overnight growth on Luria-Bertani agar containing ampicillin. Recombinant plasmids were purified and sequenced to verify code and frame.

Generation of stable cell lines. Rat-derived 5H cells were transfected using JetPEI reagent (Polyplus) according to the

Table 1. Primer Sequences in Plasmid Construction

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>d3S EcoRI-XbaI sense</td>
<td>AAT TCC CTT GCT TGG TTC TCT TGC TTA GTT CAT CCA GCA GCA AGT TGA TGA T</td>
</tr>
<tr>
<td>d3S EcoRI-XbaI anti-sense</td>
<td>CTA GAT CAT CAA CTT GCT GGA TGA ACT AAG CAA GAG AAC AAA GCA AGG G</td>
</tr>
<tr>
<td>d3NS EcoRI-XbaI sense</td>
<td>AAT TCC CTT GCT TGG TTC TCT TGC TTA ATT CAT CCA GCA GCA AGT TGA TGA T</td>
</tr>
<tr>
<td>d3NS EcoRI-XbaI anti-sense</td>
<td>CTA GAT CAT CAA CTT GCT GGA TGA ATT AAG CAA GAG AAC AAA GCA AGG G</td>
</tr>
<tr>
<td>d3G1 EcoRI-XbaI sense</td>
<td>AAT TCC CTG GCC CTG CTC TCT TGC ACT GTG CCC GCT TCA GGC TGA TGA T</td>
</tr>
<tr>
<td>d3G1 EcoRI-XbaI anti-sense</td>
<td>CTA GAT CAT CAG GCT GAA GCG GGC ACA GTC AGG CAA GAG AGC AGG GCC AGG G</td>
</tr>
<tr>
<td>d2-d3 EcoRI sense</td>
<td>GTG CGA ATT CGA ACT TGG GTA AAG TCA TCG</td>
</tr>
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Figure 1. Validation that domain 3 (d3) of hepatitis C virus (HCV) expresses stably transfected cells. A, Schematic of green fluorescent protein (GFP) control and GFP-d3 fusions, with the exact sequence corresponding to each of the different d3 constructs tested: steatosis-associated d3 (d3S), genotype 1 d3 (d3G1), and non–steatosis-associated d3 (d3NS). B, Western blot analysis of lysates prepared from cell lines stably expressing GFP and GFP-d3 fusions by means of primary antibodies against both GFP and β-actin (loading control).
Figure 2. Evidence that domain 3 (d3) is sufficient for lipid accumulation. A, High-power (100× magnification) fields from d3-expressing stable cells. Cell lines expressing green fluorescent protein (GFP) and cell lines that expressed a fusion of GFP with steatosis-associated d3 (d3S) were analyzed by means of fluorescence microscopy and oil red O (ORO) staining. GFP-expressing cells had bright green fluorescent staining and scattered, small lipid droplets on ORO staining. In contrast, GFP-d3S cells had slightly duller staining distributed throughout the cell with large lipid droplets or aggregates (white arrow). B, Results of analysis for ORO content by use of MetaMorph software. ORO content was divided by nuclear content to control for the number of cells within a field. d3G1, genotype 1 d3; d3NS, non–steatosis-associated d3.

manufacturer’s recommended protocol. After 24 h, cells were transferred to a 150-mm dish and incubated with medium containing 2 mg/mL G418 for selection. Subsequent colonies were isolated and maintained in medium containing 1 mg/mL G418.

**Fluorescent microscopy and oil red O (ORO) staining.** The GFP-core construct cell lines were passaged into 4-well chamber slides and grown overnight. Cells were then fixed and stained with ORO as described elsewhere [28]. In brief, cells were washed twice in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min at 37°C, permeabilized with 0.1% Triton-X in PBS for 5 min, and washed and stained with 4',6-diamidino-2-phenylindol (DAPI) in methanol (1:1000 concentration) for 3 min at 25°C. Cells were washed with PBS 2 times and with propylene glycol 3 times for 5 min each. ORO stain in propylene glycol (Newcomer Supply) was applied to cells for 7 min, and then cells were washed in 85% propylene glycol for 3 min. Cells underwent distilled water rinse twice and were mounted using PBS in glycerol at a 1:1 concentration. Slides were examined using an Axiovert 200 microscope (Carl Zeiss) with epifluorescent illumination, and images were recorded using an AxioCam high-resolution camera (Carl Zeiss) and Axiovision software (version 4.4; Carl Zeiss), with the same settings used for all photographs.

**ORO quantitation using MetaMorph software (Molecular Devices).** For each stable cell line, 20 contiguous high-power (100× magnification) fields were photographed for analysis. We used MetaMorph software to compare the amount of ORO content in each cell line as described elsewhere [28]. First we analyzed the entire field by applying a blue threshold to measure the percentage of the field with DAPI fluorescence, and then we analyzed the corresponding bright-field image by applying a red threshold to analyze the percentage of the field with ORO staining. We divided the percentage of the field with ORO stain by the percentage of the field with DAPI fluorescence to control for the number of cells in a given field. This was done for each stable cell line; then means and standard deviations were calculated, and the results were compared using a 2-tailed Student t test [31].

**Triglyceride assay.** Cells were maintained in a 150-mm dish...
Antibody staining was performed for 30 min at 37°C. Staining was performed as described above; primary antibodies goat anti–mouse antibody conjugated to Alexa Fluor 594 (Molecular Probes–Invitrogen) for 30 min at 37°C. 2 PBS washes, the staining procedure proceeded as described above with DAPI stain.

was carefully collected. The lower phase was allowed to air-dry until confluence and then collected on the same day on which the experiment was performed. The cells were washed twice with PBS, 400 μL of PBS was added, the cells were collected by means of a cell scraper, and 10 μL of cells were saved for measuring protein concentration. For each sample, 100 μg of total protein was used to detect the triglyceride level. To extract lipid, 1.5 mL of chloroform in methanol (1:2 concentration) was added, and the samples were incubated at room temperature for 2 min, which was followed by vortex for 30 s. Then 500 μL of chloroform was added, which was followed by vortex for 30 s, and then 500 μL of PBS was added, which was followed by another vortex for 30 s. Samples were then centrifuged at 1000 g for 10 min at room temperature, and the lower phase was carefully collected. The lower phase was allowed to air-dry overnight, and the next day a 50-μL mixture (t-butanol, Triton X-114, and methanol at a 9:4:2 concentration) was added to redissolve the dried lipids. The triglyceride level was measured at 540 nm using a Serum Triglyceride Determination kit (Sigma-Aldrich) according to the manufacturer’s instructions.

Immunofluorescence. Antibodies to adipose differentiation–related protein (ADRP), calnexin, Golgi 58k protein, and catalase (Abcam) were used for immunofluorescence experiments. Staining was performed as described above; primary antibody staining was performed for 30 min at 37°C, which was followed by 2 PBS washes and then staining with a secondary goat anti–mouse antibody conjugated to Alexa Fluor 594 (Molecular Probes–Invitrogen) for 30 min at 37°C. After 2 PBS washes, the staining procedure proceeded as described above with DAPI stain.

Endoplasmic reticulum (ER) stress and Western blot analysis. Untransfected 5H cells were used as a negative control for ER stress, and untrasfected cells that were incubated with 10 μg/mL tunicamycin for 24 h were used as a positive control. All cell lysates were prepared using Passive Lysis buffer (Promega) and subjected to immunoblot analysis by use of the following primary antibodies: GFP; glucose-regulated protein (GRP) 78; C/EBP homologous protein (CHOP), also known as GRP-94 (Abcam); β-actin (GenScript); and the secondary antibodies goat anti–mouse horseradish peroxidase and donkey anti–goat horseradish peroxidase (GenScript).

Transient transfections. Huh7.5 cells (provided by Dr Shelton Bradrick, Duke University Medical Center, Durham, NC) were transfected using JetPEI reagent (Polyplus) in a 4-well chamber slide. After 24 h, cells were stained according to the immunofluorescence protocol described above.

## RESULTS

Generation of stable cells expressing GFP fusions. Because all commercially available HCV core antibodies recognize epitopes within domains 1 and 2, we made d3-GFP fusion constructs so that we could independently monitor for the presence and localization of our d3 constructs. It is important to indicate that there are very few amino acid changes between the constructs and they are at fixed positions (Figure 1A).

We generated stable cell lines using rat liver 5H cells, which have low baseline lipid levels and are therefore a good system for our prior and ongoing experiments. Huh7 and HepG2 cells contain too much lipid at baseline to allow for analysis of lipid accumulation. As demonstrated in Figure 1B, stably transfected cells that expressed any of the constructs (control GFP, d3S, d3G1, or d3NS) all had equivalent levels of protein expression.

d3 is sufficient for lipid accumulation. We used fluorescence microscopy to examine the alterations in lipid metabolism after d3 fusion protein expression. Control GFP cells had small scattered lipid droplets; this result was consistent with

![Figure 4](image-url) Evidence that domain 3 (d3) constructs do not cause lipid accumulation by inducing endoplasmic reticulum (ER) stress. Cell lysates underwent immunoblot analysis for markers of ER stress (glucose-regulated protein [GRP-78] and C/EBP homologous protein [CHOP]) from the following: untransfected 5H cells (negative control) (minus sign), untransfected cells incubated with 10 μg/mL tunicamycin (positive control) (plus sign), green fluorescent protein (GFP), and GFP fusions with steatosis-associated d3 (d3S), genotype 1 d3 (d3G1), and non–steatosis-associated d3 (d3NS). We used β-actin as a loading control.
HCV and Lipid Accumulation

Figure 5. Evidence that domain 3 (d3) does not colocalize with lipid droplets. Stable cell lines expressing green fluorescent protein (GFP) and GFP fusions with steatosis-associated d3 (d3S) and domain 2 (d2) retained with d3 (d2-d3) were analyzed via immunofluorescence by use of the antibody to adipose differentiation-related protein (ADRP), a marker for lipid droplets.

our previous experiments in transiently transfected cells (Figure 2A) [28]. Cells expressing the d3S construct had GFP fluorescence throughout the cytoplasm and large lipid droplets. These droplets usually had a perinuclear distribution. When we examined cells that expressed the d3G1 and d3NS constructs, we found that these cells also had these large lipid droplets but that the droplets were less numerous.

We used 2 approaches to analyze the apparent differences in lipid content between the d3 constructs. The first approach was based on image analysis by use of MetaMorph software. We have used MetaMorph in the past and have validated its use for the quantitation of ORO content in a high-power field [28]. After statistical analysis of 20 high-power fields per construct, the d3S-expressing cells were found to have almost 2-fold more ORO content than did the d3NS-expressing cells and 40% more ORO content than did the d3G1-expressing cells (Figure 2B).

The second method of lipid quantitation was direct measurement of triglyceride content in the stable cells. The results mirrored the differences that were observed in the MetaMorph analysis. The d3S-expressing cells had 2-fold more triglyceride content than did the d3NS-expressing cells and ~30% more triglyceride content than did the d3G1-expressing cells (Figure 3).

Markers of ER stress are not elevated in d3-expressing cells. Given that d3 is the “signal peptide” and has been shown to localize to the ER membrane, we sought to evaluate the contribution of ER stress to lipid accumulation caused by d3 [32–34]. We evaluated 2 markers of ER stress, GRP-78 (BiP) and CHOP (GRP-94), in our stable cell lines that expressed d3 constructs. We incubated 5H cells with 10 μg/mL tunicamycin as a positive control. As shown in Figure 4, GRP-78 expression was increased in tunicamycin-treated cells but equivalent between the control and all d3-expressing cells. The same was true for CHOP.

d3 does not colocalize with lipid droplets while causing lipid accumulation. Because we established that d3 was sufficient for lipid accumulation, we next sought to determine the intracellular compartment where the lipids were accumulating. Our previous work and that of others has shown that core protein localizes to lipid droplets and that the residues that are responsible for this localization are within d2 [28, 35]. An unresolved issue is how lipid droplet localization and lipid accumulation are related, if at all. Using ADRP as a marker for lipid droplets, we found that d3 did not colocalize with these large droplets, regardless of which d3 construct was used, as illustrated by the lack of fluorescent overlap (Figure 5), but that d3 was able to cause significant lipid accumulation. We compared the d3 construct to a construct in which d2 was retained with d3 (d2-d3), which should restore the ability to localize to lipid droplets. We observed almost complete overlap of GFP and ADRP staining, which indicated colocalization of this d2-d3 construct with lipid droplets.

d3 shows partial colocalization with the Golgi apparatus. After observing the initial results of significant lipid accumulation with d3 and the localization away from the lipid droplet, we sought to determine where in the cell d3 may be mediating this effect. We chose to analyze markers for the ER, the Golgi apparatus, and the peroxisome because of their importance in various phases of lipid metabolism. Analysis of stable cells indicated a partial colocalization of d3 with the Golgi apparatus, on the basis of the overlap of fluorescent staining (Figure 6A). There was little or no overlap observed with ER or peroxisomal markers. There were no differences in localization between the d3S, d3G1, or d3NS constructs (data not shown).
Figure 6. A, Evidence that domain 3 (d3) demonstrates partial colocalization with Golgi apparatus. Transiently transfected Huh7.5 cells that stably expressed a fusion of green fluorescent protein (GFP) and steatosis-associated d3 (d3S) were analyzed by means of immunofluorescence with the following subcellular markers: calnexin (endoplasmic reticulum [ER]), Golgi 58k protein (Golgi apparatus), and catalase (peroxisome). Staining with the ER and peroxisomal markers yielded no detectable overlap in fluorescence. Staining with the Golgi marker yielded a thin rim of yellow overlap staining in the perinuclear area as well as around a large intracellular vesicular body (white arrows). B, Transiently transfected Huh7.5 cells expressing GFP-d3S stained with the Golgi marker as stated above. Similar to the 5H stably expressing d3S cells, there was a thin rim of yellow overlap fluorescence in the perinuclear region as well as around a large intracellular vesicular body (white arrows). DAPI, 4’,6-diamidino-2-phenylindol; PE 2’Ab, phycoerythrin secondary antibody.

To confirm this finding, we repeated this experiment with the use of Huh7.5 cells that were transiently transfected with the d3S construct. Again we observed partial colocalization of the d3 construct with the Golgi apparatus (Figure 6B).

DISCUSSION

In this study, we have shown that HCV core d3 is sufficient for intracellular lipid accumulation. GFP fusion constructs of d3 alone led to large lipid-containing droplets or aggregates, and the amount of ORO staining was highest in the d3S construct and lowest in the d3NS construct. These results were substantiated by the statistically significantly higher triglyceride accumulation that was observed with the d3S construct compared to the d3NS construct. As determined by our ADRP staining, d3 does not colocalize with lipid droplets to mediate this effect but rather exhibits partial colocalization with the Golgi apparatus in both the stable rat-derived liver cells and the human-derived Huh7.5 cells.

These results have several implications. The first is that the previously attributed role of d3 has been expanded. Most studies have focused on the cleavage events that occur between d2 and d3 and between d3 and the E1 glycoprotein [32, 33, 36]. Specific investigations have previously mutated these cleavage sites and stressed the importance of proper cleavage for virus assembly and release [37–39]. Our previous work, which defined polymorphisms within d3 and subsequently used site-specific mutagenesis, provided cause for reconsideration [28]. This work provides further evidence that d3 mediates much more than just cleavage events.

The second implication is that the phenomena of lipid droplet binding and lipid accumulation have been further separated. Many of the previous studies have focused on the importance of regions within d2 for lipid droplet binding [25, 35]. In our previous study, we hypothesized that these 2 phenomena may be mediated by 2 distinct regions of the core protein. This study provides specific support of our hypothesis by showing that d3 constructs alone caused significant lipid accumulation in the absence of colocalization to the lipid droplet but that d3 colocalized to lipid droplets when combined with d2. These results are in contrast to the results of other studies that described the importance of residue 164 and phenylalanine, which is a hallmark of genotype 3 constructs, in causing the upreg-
ulation of fatty acid synthase [40, 41]. Our previous data made us skeptical of the importance of this result, given that steatosis and nonsteatosis isolates both had the F164 residue yet showed statistically significant differences in the amount of lipid accumulating. We hypothesize that this residue may serve to maintain the lipid content of droplets already formed but that it is not likely to be the primary cause of lipid accumulation.

The third implication is the apparent importance of seemingly subtle sequence differences within d3. The nonsteatosis construct, which was a genotype 3 isolate that differed by only a single amino acid at position 186 (isoleucine instead of valine), still caused lipid to accumulate but at approximately one-half the amount seen with the steatosis construct. The lipid accumulation for the genotype 1 construct was between that of the steatosis construct and that of the nonsteatosis construct, but it was closer to that of the nonsteatosis construct. We interpret these results as support for the selection over time of sequences that maintain the hydrophobic helix formation, which is required for proper cleavage at the proximal and distal ends, and that also allow for enough lipids to accumulate that viral replication is enabled and assembly occurs. Alanine scanning by means of the JFH-1 system through this region of core protein had minimal effect on viral titers, which supports our hypothesis because alanine substitution would preserve the helix structure [42]. Some sequences clearly exceed that minimum requirement, which we hypothesize to lead to the virus-induced steatosis observed in patients with chronic infection. Given the results of previous studies and the role of d3 as the “signal peptide,” we were surprised by the lack of colocalization with the ER. However, the partial colocalization of d3 with the Golgi apparatus may provide insights about a possible mechanism. We hypothesize that d3 alters the trafficking of triglycerides and other lipids by means of a hitherto unidentified mechanism and allows them to accumulate. More specific studies on the natural life cycle of d3 after cleavage and specific studies on lipid trafficking will help to address this issue.

Our results should be analyzed in light of certain limitations. First, although we are using GFP fusion constructs, which might raise concern about the context of our results, our d3-expressing cells resemble Huh7.5 cells that express a mutant core protein with an altered cleavage site between d2 and d3 [39]. These cells had increased lipid amounts and had multiple large vacuolar structures that were similar to those seen in our d3-expressing cells. Second, this system lacks the context of other viral proteins and the entire viral life cycle. These results, however, still are consistent with previous results, given the accumulation of free cholesterol with d3 constructs and the genotype differences.

In conclusion, we have demonstrated that HCV core d3 is sufficient for intracellular lipid accumulation and further underlined the importance of subtle sequence changes in the ability of d3 to cause lipid accumulation. These data provide further evidence for an expanded role of d3 in the viral life cycle beyond simple cleavage events and possibly indicate that altered trafficking is the most likely or dominant role. Elucidation of the specific mechanisms of lipid accumulation may serve to uncover a pan-genotype drug target that would serve to disrupt an early step in the viral life cycle.

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References