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# Hepatitis C Virus Glycoproteins Mediate Low pH-dependent Membrane Fusion with Liposomes*

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**It has been suggested that the hepatitis C virus (HCV) infects host cells through a pH-dependent internalization mechanism, but the steps leading from virus attachment to the fusion of viral and cellular membranes remain uncharacterized. Here we studied the mechanism underlying the HCV fusion process *in vitro* using liposomes and our recently described HCV pseudoparticles (pp) bearing functional E1E2 envelope glycoproteins. The fusion of HCVpp with liposomes was monitored with fluorescent probes incorporated into either the HCVpp or the liposomes. To validate these assays, pseudoparticles bearing either the hemagglutinin of the influenza virus or the amphotropic glycoprotein of murine leukemia virus were used as models for pH-dependent and pH-independent entry, respectively. The use of assays based either on fusion-induced dequenching of fluorescent probes or on reporter systems, which produce fluorescence when the virus and liposome contents are mixed, allowed us to demonstrate that HCVpp mediated a complete fusion process, leading to the merging of both membrane leaflets and to the mixing of the internal contents of pseudoparticle and liposome. This HCVpp-mediated fusion was dependent on low pH, with a threshold of 6.3 and an optimum at about 5.5. Fusion was temperature-dependent and did not require any protein or receptor at the surface of the target liposomes. Most interestingly, fusion was facilitated by the presence of cholesterol in the target membrane. These findings clearly indicate that HCV infection is mediated by a pH-dependent membrane fusion process. This paves the way for future studies of the mechanisms underlying HCV membrane fusion.**

Hepatitis C virus (HCV)[2] belongs to the genus *Hepacivirus* of the Flaviviridae family (1) and has infected some 170 million people worldwide (2). In the majority of HCV-infected patients, the progression to chronic disease is associated with an increased risk of liver diseases and hepatocellular carcinoma. No vaccine is presently available, and the current treatment for chronic hepatitis C (a combination of pegylated interferon and ribavirin) has a limited efficacy (3). A more detailed understanding of the molecular mechanisms of virus entry would be beneficial to the development of novel therapeutic strategies.

The HCV genome is a positive-stranded RNA encoding a precursor polyprotein of about 3,000 amino acids. This polyprotein is cleaved co- and post-translationally to generate 10 viral proteins, classified into structural (capsid protein and the envelope glycoproteins E1 and E2) and nonstructural proteins (NS2 to NS5B), separated by the membrane ion channel polypeptide p7 (1, 4). The type I transmembrane envelope glycoproteins E1 and E2 form noncovalently linked heterodimers in the endoplasmic reticulum and are highly glycosylated, containing up to 6 and 11 potential glycosylation sites, respectively (5–8). Studies of the HCV life cycle have been hampered by the lack of an efficient and reliable cell culture system to produce and isolate the functional virus. Most recently, *in vitro* models of hepatitis C virion production have been designed (9–11). However, these systems are not easy to use to study viral fusion *in vitro*, because of safety restrictions and because of the fact that wild type HCV particle production is tightly coupled to viral replication, a process that is very sensitive to mutations or alterations of the genomic sequence. We and others have shown previously that HCVpp, a model system for HCV cell entry based on retroviral or lentiviral core particles displaying HCV E1 and E2, closely mimic the early entry steps of the wild type virus (12–19). Indeed, HCVpp display a preferential tropism for hepatic cells, require the presence of both glycoproteins for activity, and can be neutralized by HCV patient sera. Furthermore, HCVpp offer technical flexibility, which we have exploited in our fusion studies by incorporating different glycoproteins, labeled probes, and marker genes into the viral core particles.

Over the last 15 years, a number of different approaches have implicated several molecules in HCV cell attachment or fusion. HCV has been shown to interact with C-type lectins L-SIGN and DC-SIGN, and it was proposed that the virus may utilize these interactions to cross the liver epithelium to access hepatocytes (20–22). Among the cellular factors mediating HCV entry into hepatocytes are tetraspanin CD81 (13, 20, 23, 24), the human scavenger receptor SR-B1 (23, 25), and likely the receptor for low density lipoproteins (26, 27). However, the precise role of each receptor in HCV entry is still unclear. We and others have shown that HCV entry occurs in a pH-dependent manner via endocytosis, as was shown for other Flaviviridae. Indeed, the use of drugs that inhibit endosomal acidification efficiently blocked HCVpp infection (12, 13, 17). Furthermore, low pH treatment of HCVpp led to the exposure of new epitopes in E2 (28), supporting the notion that low pH induces conformational rearrangements in HCV glycoproteins, eventually leading to fusion with the endosome membrane. This would be consistent with a membrane fusion mechanism similar to that described for the glycoproteins of Flaviviruses, such as tick-borne encephalitis

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[2] The abbreviations used are: HCV, hepatitis C virus; pp, pseudoparticles; HA, hemagglutinin; MLV, murine leukemia virus; PC, phosphatidylcholine; SFV, Semliki Forest virus; TBEV, tick-borne encephalitis virus; chol, cholesterol; PBS, phosphate-buffered saline; $R_{18}$, octadecyl rhodamine B chloride; HIV-1, human immunodeficiency virus, type 1; BlaM, β-lactamase; Vpr, viral protein R; env, envelope.

virus (TBEV) (29, 30), dengue virus (13, 31) and West Nile virus (32, 34). In the Flaviviridae family, it is known that low pH triggers glycoprotein conformational changes, leading to the formation of a fusion-competent trimer, in which the fusion peptide becomes exposed and inserts into the target (endosomal) membrane to initiate fusion. This process has been described for Flaviviruses such as TBEV (33) to be enhanced by the presence of cholesterol in the target membrane. Cholesterol has also been reported to enhance entry, but not the fusion process itself, of many other pH-dependent viruses (29). In contrast, for alphaviruses such as the Semliki Forest (SFV) and Sindbis viruses, the presence of cholesterol in the target membrane is an absolute requirement for fusion to occur (35–39).

In this study, we investigated the membrane fusion properties of HCVpp bearing functional E1 and E2 HCV glycoproteins, with respect to pH, temperature, viral dose, and the presence of cholesterol. We used a liposomal membrane system, composed of phosphatidylcholine (PC) liposomes with or without cholesterol and fluorescent probes ($R_{18}$, CCF2) incorporated into either the particles or the liposomes. We demonstrated that HCVpp-mediated membrane fusion is a full fusion mechanism, leading to both lipid mixing and contents mixing of HCVpp and liposome. The fusion is dependent on the presence of E1 and E2 glycoproteins, is temperature-dependent, occurs optimally at low pH (about 5.5), and does not require the presence of a protein or carbohydrate receptor in the target membrane. Although HCVpp-mediated fusion does not strictly rely on the presence of cholesterol, it is facilitated by its presence in the target membrane.

## EXPERIMENTAL PROCEDURES

### Chemicals

Phosphatidylcholine from egg yolk (99% pure), cholesterol (chol, 99% pure), and Triton X-100 were from Sigma. Phospholipid oxidation was routinely checked by spectrophotometry. Octadecyl rhodamine B chloride ($R_{18}$) was from Molecular Probes, and CCF2 (CCF2-FA; free acid form) was purchased from Invitrogen.

### Preparation of Liposomes

All liposomes were large unilamellar vesicles (100 or 400 nm), consisting of PC or PC:chol (70:30 molar ratio). $R_{18}$-labeled liposomes were obtained by mixing $R_{18}$ and lipids as ethanol and chloroform solutions, respectively (5 mol % $R_{18}$ final (40)), and liposomes were prepared by extrusion over polycarbonate filters in phosphate-buffered saline (PBS), pH 7.4 (41). CCF2-loaded liposomes were obtained by resuspending the lipid film into a 100 $\mu$M CCF2-FA solution in 25 mM Hepes, 150 mM NaCl, pH 7.5; unencapsulated CCF2 was removed by gel filtration over a PD-10 column.

### Vector Constructs

The CMV-Gag-Pol murine leukemia virus (MLV) packaging construct, encoding the MLV *gag* and *pol* genes, and the MLV-GFP plasmid, encoding an MLV-based transfer vector containing a CMV-GFP internal transcriptional unit, were described previously (12). The pCMV 8.91 HIV-1 Gag-Pol packaging construct contains all HIV-1 genes except *env* encoding the envelope glycoproteins and *nef* (kind gift of Dr. D. Trono). The phCMV-HA (42) and NA expression vectors encode the hemagglutinin and neuraminidase of fowl plague virus, respectively. The phCMV-MLV-A vector encodes the envelope glycoprotein Env of the amphotropic MLV (43). The phCMV-E1E2-HCV (12) encodes both HCV E1 and E2 glycoproteins of genotype 1a, strain H77 (AF009606).

### Preparation of Pseudoparticles

Pseudoparticles were generated as described previously (12). Briefly, 293T cells were transfected using a calcium phosphate-based transfection kit with three expression vectors encoding an envelope glycoprotein, viral core components, and a viral genome displaying a green fluorescent protein marker gene. Particles produced without any envelope expression construct, and consequently not infectious, were denoted "no env pp." To incorporate specifically $\beta$-lactamase (BlaM) into HIV-based particles, a chimeric protein encoding BlaM linked to the N terminus of the HIV-1 viral protein R (Vpr) was co-expressed with the other HIV viral vectors (44, 45). Cell supernatants containing pseudoparticles were harvested 36 h after transfection and concentrated 100-fold by centrifugation through 2 ml of a 25% sucrose cushion at 100,000 $\times$ *g* in a Beckman SW 41 rotor for 120 min at 4 °C, followed by resuspension in PBS. Infectious titers of the concentrated supernatants were then determined by infection of Huh7 cells followed by fluorescence-activated cell sorter analysis. The titers of the different concentrated pseudo-particle preparations were $\sim 2 \times 10^6$ IU/ml, $3 \times 10^8$ IU/ml, and $5 \times 10^8$ IU/ml for HCVpp, MLVpp, and HApp, respectively. Viral pellets and cell lysates of viral producer cells were subjected to immunoblot analysis using a mouse anti-HCV E2 (H52 (46)), a mouse anti-HCV E1 (A4 (47)), and a goat anti-glycoprotein 70 (MLV-A SU) serum (ViroMed Biosafety Laboratories, Camden, NJ). HA was detected with a rabbit polyclonal anti-HA H7 strain antibody (anti-Kp-Ro Rostock cl96-11, kindly provided by W. Garten, Marburg, Germany). To compare the amounts of viruses, the blots were probed with antibodies against the capsid proteins of HIV and MLV as follows: the mouse anti-p24 of HIV (antibody AG3.0, obtained through the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH, from Dr. Jonathan Allan) and the anti-p30 of MLV (goat antiserum raised against the Rauscher-MuLV p30; ViroMed). Monoclonal anti-$\beta$-lactamase antibody was from Chemicon International (AB3738), and the rabbit polyclonal anti-Vpr antibody was a kind gift from Dr. Warner Greene (45).

### Fusion Assays

*Lipid Mixing*—$R_{18}$ was incorporated into either the liposomes or the pseudoparticles at self-quenching concentrations (40). Unincorporated $R_{18}$ was removed by pelleting the pseudoparticles for 1 h at 100,000 $\times$ *g* and 4 °C in a Beckman SW 41 rotor. Lipid mixing between pseudoparticles and liposomes was monitored as the dequenching of $R_{18}$. Briefly, $R_{18}$-labeled liposomes (final lipid concentration, 15 $\mu$M) were added to a 37 °C-thermostated cuvette containing pseudoparticles in PBS, pH 7.4. After temperature equilibration, fusion kinetics were recorded on an SLM Aminco 8000 spectrofluorimeter, over a 30-min time period, at $\lambda_{exc}$ = 560 nm and $\lambda_{em}$ = 590 nm. Maximal $R_{18}$ dequenching was measured after the addition of 0.1% Triton X-100 (final concentration) to the cuvette. The same procedure was used for lipid mixing assays with $R_{18}$-labeled pseudoparticles (2 nmol of $R_{18}$ per 100 $\mu$l of particles) and unlabeled liposomes (15 $\mu$M). The initial rates of fusion were taken as the value of the slope of the tangent, drawn to the steepest part of the fusion kinetics.

*Internal Contents Mixing*—This assay is based upon the cleavage of a fluorogenic substrate, CCF2 (encapsulated into liposomes) by $\beta$-lactamase, where BlaM is incorporated into viral core particles in the form of a BlaM-Vpr chimera (44) and CCF2 is incorporated in the lumen of liposomes. CCF2 is a cephalosporin-derived molecule bearing coumarin-derived and fluorescein moieties in close proximity, because of their association with the $\beta$-lactam ring (48). In CCF2, the absorption spectrum of the fluorescence acceptor (fluorescein) and the emission spec-

trum of the donor (coumarin) overlap. In this configuration an efficient internal fluorescence resonance energy transfer reaction takes place, and the acceptor fluorescence can be observed at 520 nm. When the enzyme BlaM cleaves the $\beta$-lactam ring of CCF2, the coumarin moiety is relieved from the internal fluorescence resonance energy transfer, and its fluorescence increases at 450 nm, whereas fluorescein fluorescence decreases at 520 nm. When aqueous liposome and pseudoparticle contents coalesce as a result of fusion, $\beta$-lactamase cleaves CCF2 into a nonfluorescent fluorescein-derived moiety and a fluorescent coumarin-based moiety exhibiting a $\lambda_{exc}$ of 409 nm and a $\lambda_{em}$ of 450 nm. The final concentration of CCF2 used in the assay was 25 $\mu$M, and the total lipid concentration was 150 $\mu$M. Contents mixing was visualized both by recording the emission spectra of CCF2 between 420 and 560 nm ($\lambda_{exc}$ = 409 nm) and by monitoring the increase of coumarin fluorescence kinetically at $\lambda_{exc}$ = 409 nm and $\lambda_{em}$ = 450 nm.

## RESULTS

*Fusion of HCVpp with Liposomes Assessed by Lipid Mixing*—Fusion of a virus with the membrane of its target cell implies initial destabilization of viral and cellular membranes, which leads to mixing of viral and cellular lipids (lipid mixing) and ultimately to the formation of a pore connecting the viral and cellular internal compartments (contents mixing). To assess the lipid mixing properties of HCVpp, we deployed two assays based upon rhodamine ($R_{18}$) fluorescence dequenching, a well documented test for viral fusion (40). We incorporated $R_{18}$ into pseudoparticles or liposomes at a surface density to which the probe is efficiently self-quenched. Upon mixing of membranes containing self-quenched $R_{18}$ with membranes devoid of the fluorophore, the dilution of the probe results in dequenching, which is directly proportional to the ratio of $R_{18}$ to total lipid. Thus lipid mixing can be followed kinetically by measuring the $R_{18}$ increase in fluorescence over time. Moreover, the fluorescence yield of $R_{18}$ is not sensitive to pH changes of the surrounding solvent and makes it suitable for membrane fusion studies over a large pH range (40).

The first approach used $R_{18}$-labeled pseudoparticles. To control the input of the different types of pseudoparticles in the fusion experiments, we normalized our assays by estimating the amount of MLV core protein (p30) in each viral preparation by immunoblotting concentrated viral supernatants with an anti-p30 antibody. Fig. 1*A* shows that the amount of core used in the assays was similar for all pseudoparticle preparations assayed in fusion (*top panel*). Immunoblot analyses of the glycoproteins (Fig. 1*A*, *bottom panels*) showed that the appropriate glycoproteins were present in each viral preparation.

For the lipid mixing reaction, PC liposomes were prepared by extrusion through a 100-nm pore, a diameter comparable with that of the pseudoparticles (49). Upon the addition of $R_{18}$-labeled HCVpp to unlabeled liposomes, $R_{18}$ dequenching was observed only when the pH was decreased to 5.0 (Fig. 1*B*, *HCVpp*). At pH 7.4 (Fig. 1*C*, *curve a*), even after long incubation times, no significant dequenching could be detected. HCVpp behaved similarly to HApp bearing the hemagglutinin of the influenza fowl plague virus, a well known prototype for a strictly low pH-dependent fusion protein. Indeed as illustrated in Fig. 1*B* (representative curves of five experiments), a rapid fluorescence dequenching was observed for HApp when the pH was decreased to 5.0, whereas no fluorescence variations occurred at pH 7.4 (Fig. 1*C*, *curve b*). This strictly pH-dependent HApp-mediated lipid mixing was comparable with reported data on the fusion of the wild type influenza virus with liposomes (40, 50). The kinetics of HApp lipid mixing are reproducibly biphasic, exhibiting a very rapid initial rate that might be due to a higher intrinsic fusogenicity of HApp compared with HCVpp. Control pseudoparticles devoid of viral glycoprotein (Fig. 1*B*, *no env pp*) or pseudoparticles bearing the amphotropic murine leukemia virus MLV glycoprotein (MLVpp), a receptor-dependent and pH-independent glycoprotein (51), did not display any lipid mixing at any pH and any amount of pseudoparticle tested (Fig. 1*C*, *curve c* and data not shown).

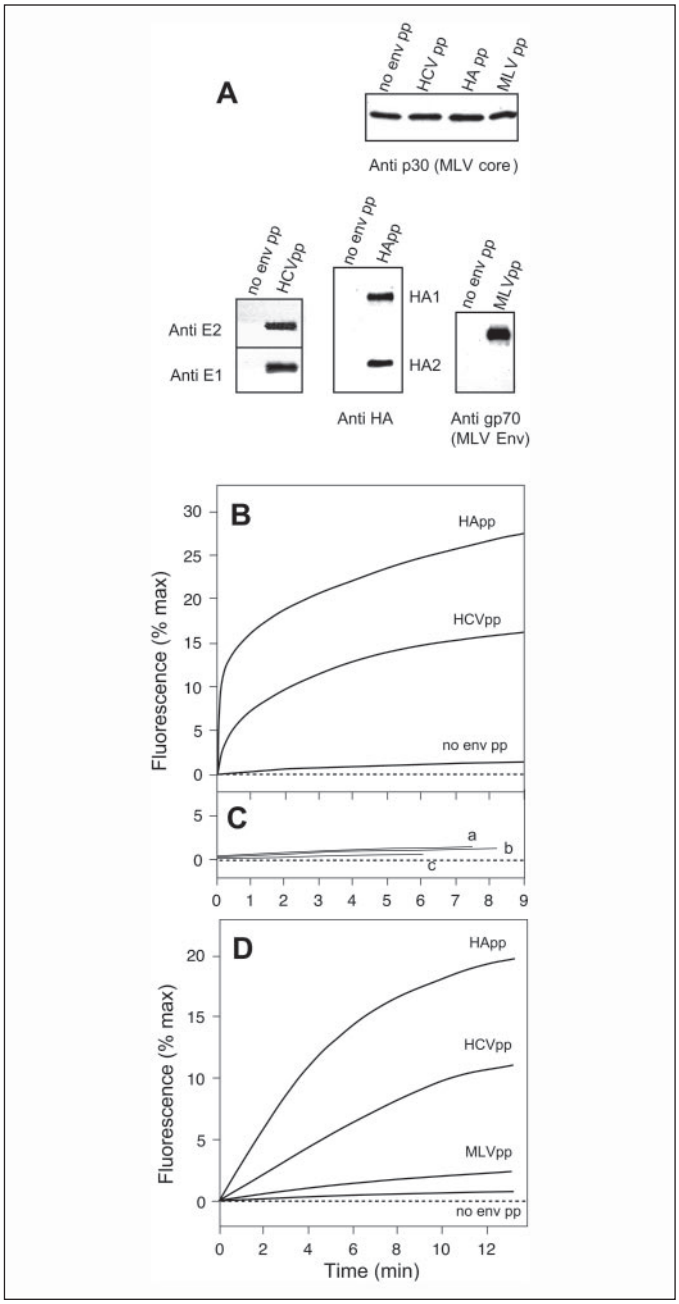


FIGURE 1. **HCVpp lipid mixing properties.** *A,* immunoblots of pseudoparticles generated with HCV E1/E2 proteins (*HCVpp*), HA (*HApp*), MLV-A Env (*MLVpp*), or in the absence of any envelope protein (*no env pp*) and probed for the envelope glycoproteins and the viral core using specific antibodies (see "Experimental Procedures"). *B* and *C,* kinetics of lipid mixing for HCVpp, HApp, and no env pp at pH 5.0 and 7.4, respectively (representative of five separate experiments); *curve a,* HCVpp; *curve b,* HApp; *curve c,* MLVpp. HCV pseudoparticles were labeled with $R_{18}$ as described under "Experimental Procedures" and incubated with PC liposomes in PBS, pH 7.4, at 37 °C. After a 2-min equilibration, lipid mixing was initiated by decreasing the pH to 5.0 (time 0) and recorded as $R_{18}$ fluorescence dequenching as a function of time. *D,* lipid mixing curves of HCVpp, HApp, MLVpp, and no env pp at pH 5.0 and 37 °C with $R_{18}$-labeled liposomes (representative of five separate experiments). Pseudoparticles (40 $\mu$l) were added in PBS, pH 7.4, containing liposomes (15 $\mu$M, final lipid concentration) labeled with $R_{18}$ as described under "Experimental Procedures." After a 2-min equilibration, the pH was decreased to 5.0 (time 0), and lipid mixing was recorded as a function of time.

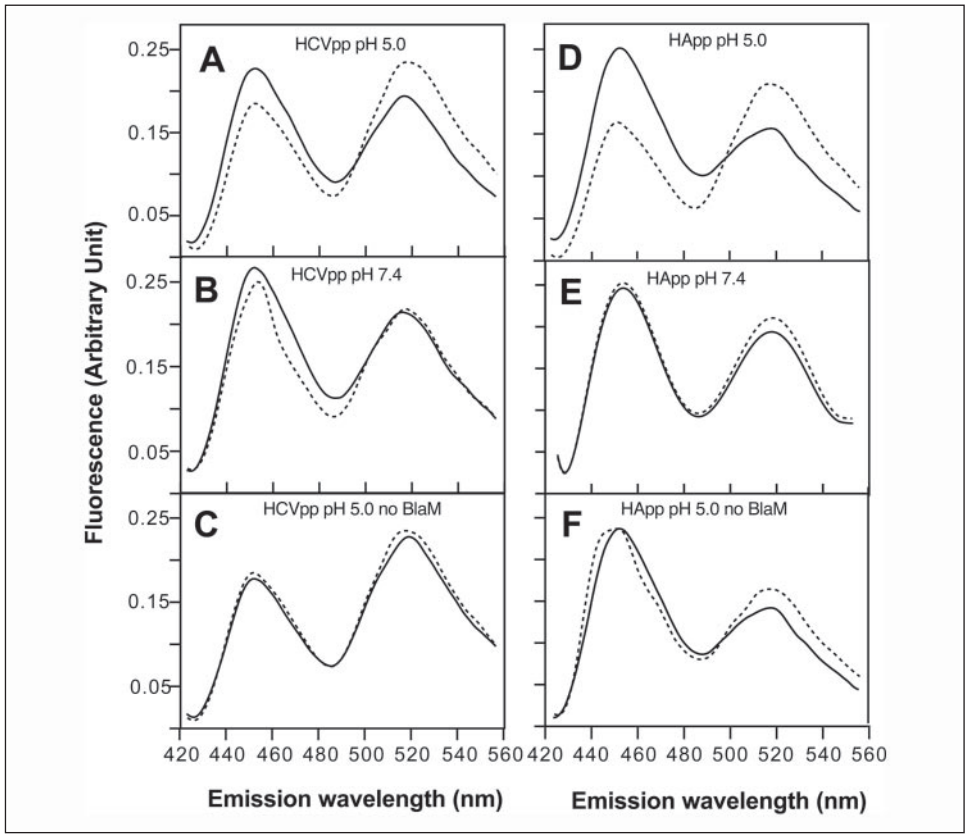


FIGURE 2. **Changes in CCF2 fluorescence as a measure of HCVpp-mediated contents mixing.** CCF2 was encapsulated into liposomes as described under "Experimental Procedures" and incubated with HIV core-based pseudoparticles containing β-lactamase (*BlaM*) fused to Vpr protein (*BlaM-Vpr*). CCF2-loaded liposomes (150 μM phospholipid and 25 μM CCF2 final) were equilibrated at 37 °C in PBS, pH 7.4, and pseudoparticles were added. Fusion was initiated by decreasing the pH to 5.0 (time 0 = $t_0$). Excitation was set at 409 nm, and emission spectra were collected between 420 and 560 nm over a 30-min time period. Cleavage of CCF2 by BlaM-Vpr occurred after mixing of luminal contents and was visualized by comparing CCF2 emission fluorescence spectra recorded at $t_0$ (*dashed line*) and after 30 min (*solid line*). A fluorescence increase of the coumarin moiety at 450 nm concomitant with a decrease in the fluorescence of the fluorescein moiety at 520 nm reveal the BlaM-dependent CCF2 cleavage efficiency of HCVpp at pH 5.0 (*A*) but not at pH 7.4 (*B*) or for HCVpp devoid of BlaM-Vpr at pH 5.0 (*C*). Similar results were obtained for HApp at pH 5.0 with (*D*) or without (*F*) BlaM-Vpr; (*E*) HApp with BlaM-Vpr at pH 7.4.

This demonstrates that lipid mixing was specifically induced by the HCV and HA glycoproteins in a pH-dependent manner and occurred in the absence of receptor on the target liposomes.

To confirm these data, we performed similar lipid mixing experiments using liposomes labeled with $R_{18}$ at self-quenching concentrations and with unlabeled pseudoparticles. When $R_{18}$-labeled liposomes were added to pseudoparticles, rapid dequenching was again observed for HCVpp and HApp, only at pH 5.0, but not at all for MLVpp or no env pp (Fig. 1*D*, representative curves of five experiments). Note however that the kinetics of lipid mixing appears to be slowed down under those conditions when compared with the kinetics recorded with $R_{18}$-labeled pseudoparticles (Fig. 1*B*). This could be due to different amounts of incorporation of $R_{18}$ into liposomes and into pseudoparticles, and/or to the fact that $R_{18}$ is in both leaflets of liposomes instead of residing only in the outer leaflet of pseudoparticles. An alternative explanation might be that $R_{18}$ dilution from protein-free liposomes into protein-containing pseudoparticle membranes is somehow impeded, compared with its dilution from viral membranes into liposomes.

*HCVpp Induce Contents Mixing, Displaying a Complete Fusion Mechanism*—By having established with $R_{18}$ that HCVpp efficiently promoted lipid mixing in a pH-dependent and concentration-dependent manner (see below Fig. 4*A*), we asked whether HCVpp-liposome fusion led to mixing of the internal contents of pseudoparticles and liposomes. For this purpose we developed a fluorescent reporter assay that exploited the incorporation of BlaM into the core structure of HIV-based pseudoparticles. As the accessory HIV protein Vpr binds to the p6 core component of the HIV-1 Gag polyprotein, heterologous proteins have been integrated into HIV virions in the form of Vpr fusion proteins. They have been successfully used to study the early events of HIV-1 and Ebola virus fusion (44, 45). In this study, we incorporated a BlaM-Vpr chimera (BlaM-Vpr) into HIV-based pseudoparticles displaying either HCV or control envelope glycoproteins. CCF2 is a fluorogenic β-lactam substrate of BlaM and is composed of two fluorescent moieties, a coumarin derivative and fluorescein. It was encapsulated into liposomes in the negatively charged free acid form, which is membrane-impermeable and cannot passively leak out of the liposomes. Upon contents mixing between pseudoparticles and liposomes, CCF2 within the liposomal lumen becomes accessible to BlaM-Vpr, and cleavage of the CCF2 dye by the BlaM protein results in a shift of the fluorescence emission spectrum of CCF2, which can be followed over time (see details under "Experimental Procedures").

In Fig. 2 we compared the emission spectra of CCF2-loaded liposomes before mixing with different pseudoparticles containing BlaM-Vpr (*dashed line*, $t_0$) with the spectra obtained after a 30-min incubation (*solid line*). HCVpp induced a decrease in the emission spectrum at 520 nm (fluorescein moiety of CCF2) concomitant with an increase at 450 nm (coumarin moiety of CCF2), only when the reaction was performed at pH 5.0 (Fig. 2*A*) but not at a neutral pH (Fig. 2*B*). When HCVpp were devoid of the BlaM-Vpr enzyme, no CCF2 cleavage could be observed, as revealed by the absence of spectral changes over time, both for the coumarin and fluorescein moieties of CCF2 (Fig. 2*C*). With HApp we also detected changes in the emission spectra of the CCF2 moieties at pH 5.0 (Fig. 2*D*). However, no change in the spectra was obtained for HApp at a neutral pH (Fig. 2*E*) or when HApp were devoid of BlaM-Vpr (Fig. 2*F*) or when MLV-based instead of HIV-based pseudoparticles were used, which are unable to incorporate BlaM-Vpr (data not shown). Leakage of BlaM or CCF2 during the course of the contents mixing reaction was ruled out by incubating BlaM-containing pseudoparticles devoid of glycoproteins with CCF2 in solution at a neutral or acidic pH (data not shown). To check for photobleaching of CCF2 during the incubation period, we continuously illuminated control CCF2-loaded

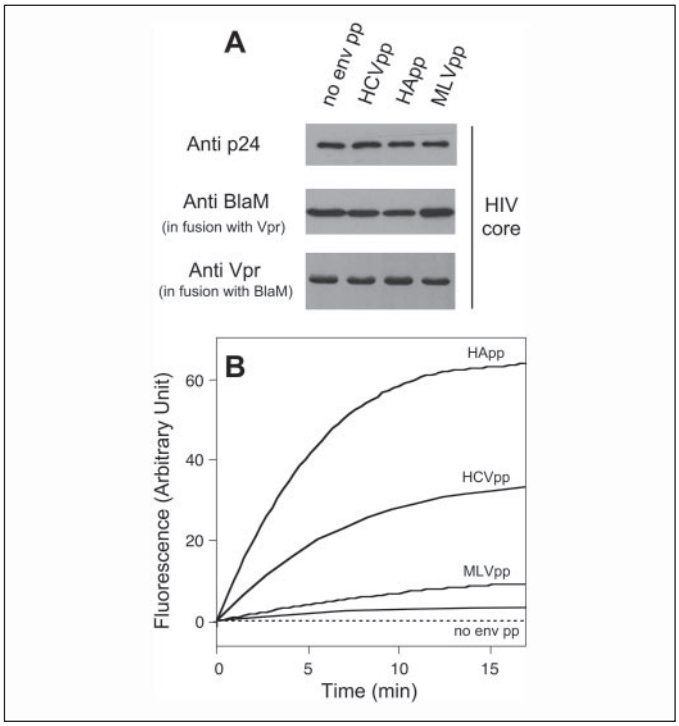


FIGURE 3. **Kinetics of BlaM-induced cleavage of CCF2 as a measure of HCVpp-mediated internal contents mixing.** *A,* immunoblots of pseudoparticles generated with HIV core proteins and harboring HCV E1/E2 proteins (*HCVpp*), or HA (*HApp*), or MLV Env (*MLVpp*), or in the absence of any envelope protein (*no env pp*), and probed for the envelope glycoproteins, the viral core p24, BlaM, and Vpr, using specific antibodies (see "Experimental Procedures"). *B,* kinetics of coumarin fluorescence. PC liposomes containing 25 $\mu$M-enclosed CCF2 were incubated in PBS, pH 7.4, at 37 °C with 40 $\mu$l of HIV core-based pseudoparticles containing BlaM-Vpr. At time 0, the pH was decreased to 5.0, and the fluorescence of the coumarin moiety was recorded at $\lambda_{em}$ 450 nm as a function of time ($\lambda_{exc}$ 409 nm).

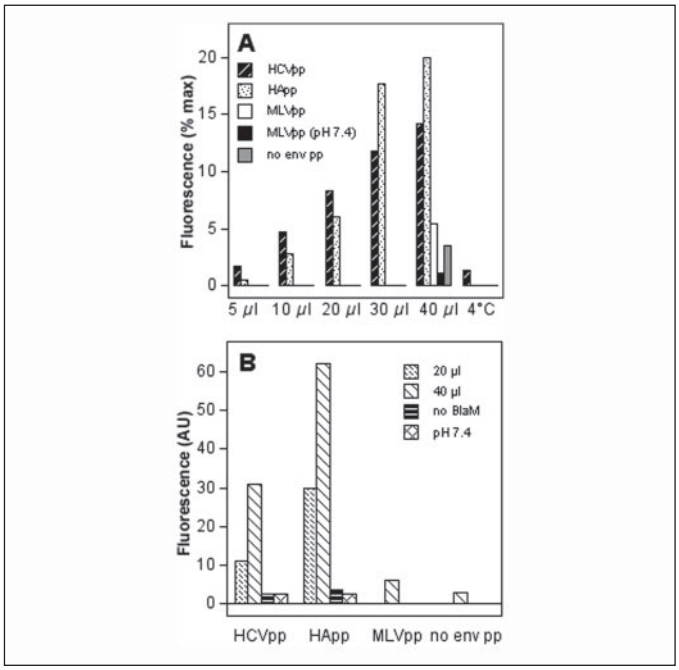


FIGURE 4. **Dose and temperature dependence of HCVpp-mediated lipid mixing.** *A,* liposomes were labeled with $R_{18}$, and fusion was measured as in Fig. 1*D*. The dose dependence of lipid mixing for increasing amounts of HCVpp (*hatched bar*), HApp (*dotted bar*), and MLVpp (*white bar*) was determined at pH 5.0 and 37 °C. Fusion at 4 °C was recorded for 40 $\mu$l of HCVpp. Fusion of MLVpp at pH 7.4 (*black bar*) and no env at pH 5.0 (*gray bar*) was recorded at 37 °C. *Bar height* corresponds to the average percentage of fluorescence recorded at the plateau, obtained after a 20 min-recording for each pseudoparticle tested. *B,* internal content mixing was measured as in Fig. 2 with CCF2 encapsulated into liposomes and HIV core-based pseudoparticles containing BlaM-Vpr. *Bar height* corresponds to the average fluorescence recorded at the plateau, observed after a 20-min recording for each pseudoparticle tested. *Light* and *dark hatched bars* correspond to 20- and 40-$\mu$l pseudoparticle preparations, respectively, tested at pH 5.0; *bars with chevrons* correspond to 40 $\mu$l of these pseudoparticles at pH 7.4; *bars with horizontal thick lines* correspond to 40 $\mu$l of pseudoparticles devoid of BlaM-Vpr and recorded at pH 5.0.

liposomes and followed their behavior kinetically, but no change in the fluorescence signal was observed (data not shown).

The pseudoparticle input for this contents mixing assay was again standardized by quantifying the viral HIV core protein p24, as well as the BlaM-Vpr in each pseudoparticle preparation, by immunoblotting with anti-p24, anti-BlaM, and anti-Vpr antibodies (Fig. 3*A*).

The kinetics corresponding to the spectral changes presented in Fig. 2 as end point values are shown in Fig. 3*B*. Upon incubation of the HCV pseudoparticles with CCF2-loaded liposomes and a pH decrease to 5.0 (Fig. 3*B*, *HCVpp*), an increase in the moiety fluorescence of the coumarin was observed, according to kinetics that are reminiscent of those of the $R_{18}$ lipid mixing assay (Fig. 1*D*). Similar behavior was observed for HApp (Fig. 3*B*, *HApp*). Note that *MLVpp* and *no env pp* of Fig. 3*B* displayed very low or no propensity, respectively, to mix their luminal content with that of liposomes, thus failing to display fusion activity, as already observed in the $R_{18}$ lipid mixing assay (Fig. 1, *B* and *C*).

Maximal CCF2 fluorescence was observed after 30 min for HCVpp and after 10 min for HApp (data not shown). Note that we follow the enzymatic turnover of CCF2 by BlaM as a reflection of coalescence of aqueous compartments. Therefore, we cannot exclude that the actual internal contents mixing is more rapid than the kinetics we observed. Nevertheless, the kinetics of the HCVpp-mediated lipid mixing (Fig. 1*D*) and of the BlaM-mediated turnover of CCF2 as a measure of HCVpp/liposome contents mixing (Fig. 3*B*) are comparable, and both events leveled off after ~20 min (average of several measurements). This demonstrates a genuine and complete fusion between HCVpp and liposomes. This observation is still strengthened by the fact that fusion extents are proportional to the amounts of viral particles, both in lipid mixing (Fig. 4*A*) and contents mixing assays (Fig. 4*B*).

Taken together, these results indicate that the HCV glycoproteins E1 and E2 induce lipid mixing as well as contents mixing and thus support full fusion activity in the absence of receptor molecules, but this requires a low pH. However, it must be noted that the extents of fusion are consistently less important with HCVpp than with HApp. This might be due to a lower efficiency of incorporation of HCV glycoproteins onto HCVpp than of HA onto HApp and/or to a lower intrinsic infectivity of HCV glycoproteins than that of HA (see also "Discussion").

*Influence of Viral Dose and Temperature on HCVpp Fusion*—To test the influence of viral dose and temperature on HCVpp fusion activity, we performed the $R_{18}$-based lipid mixing assay (Fig. 4*A*), as well as the contents mixing assay based upon the cleavage of CCF2 by BlaM-Vpr (Fig. 4*B*), with different amounts of pseudoparticles at either 4 or 37 °C. Pseudoparticles were mixed with liposomes, and fusion activity was monitored from the moment the pH was decreased to pH 5.0, for 20 min, when fusion had leveled off (data not shown). In both assays, the final levels of HCVpp were proportional to the volume of pseudoparticles added to the liposome suspension (Fig. 4, *A* and *B*). At high pseudoparticles-to-liposomes ratios (30- and 40-$\mu$l pseudoparticle volumes in Fig. 4*A*), HCVpp fusion leveled off, indicative of a saturation level in the fusion process. Similar observations could be made for HApp, in agreement with reported observations on influenza virus fusion (50). In contrast, MLVpp or no env pp mediated only low or negligible fluorescence dequenching at any pH tested and even at high pseudoparticle

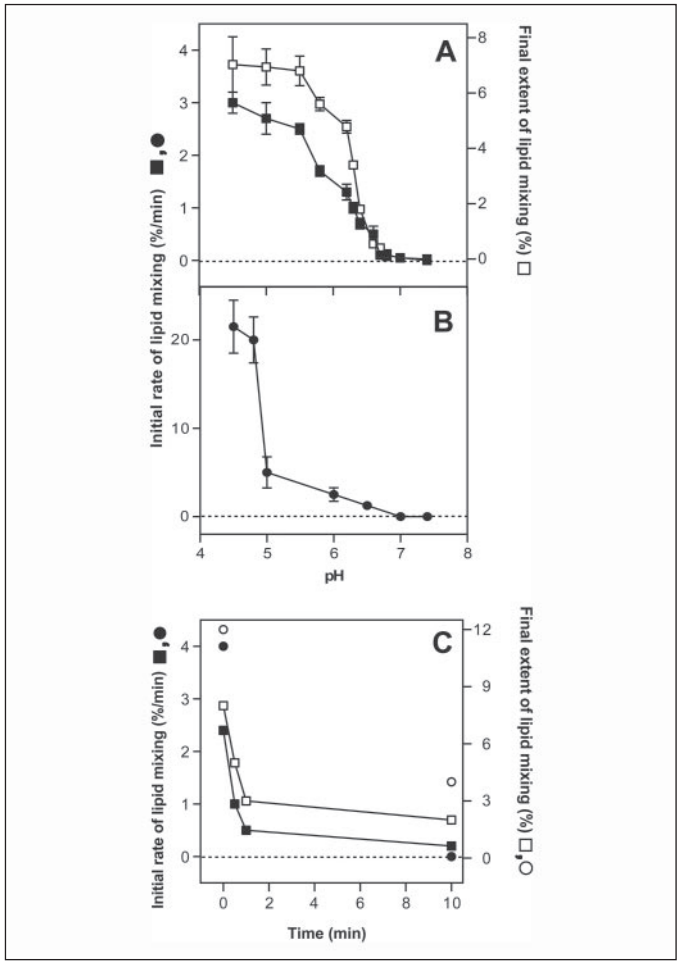


FIGURE 5. **pH dependence of lipid mixing for HCVpp (*A*) and HApp (*B*) and low pH inactivation (*C*).** Lipid mixing of pseudoparticles with $R_{18}$-labeled liposomes was measured as in Fig. 1*D* at the indicated pH. Initial rates of lipid mixing were determined for each pH from the tangents to the steepest parts of the fusion curves (*A, closed symbols*). The final extent of lipid mixing is the value of fluorescence for each pH at the 20-min time point (*A, open symbols*). Each point represents the average value of three separate measurements. *B,* initial rates of lipid mixing for HApp at indicated pH for three separate experiments. *C,* HCVpp (*squares*) and HApp (*circles*) were pre-exposed to pH 5 for the indicated time in the absence of liposomes. $R_{18}$-labeled liposomes were then added, and lipid mixing was recorded as described above. Initial rates (*closed symbols*) and final extent of lipid mixing (*open symbols*) were plotted as a function of time of preincubation at low pH.

levels. Furthermore, monitoring of HCVpp-induced $R_{18}$ dequenching at 4 °C (Fig. 4*A*) did not lead to significant changes in the fluorescence signal over the time course of the experiment, even at acidic pH. Thus HCV E1/E2 glycoprotein-mediated fusion occurs in a dose-dependent fashion and is a process that requires energy for activation, as it only occurs at high temperatures.

*pH Dependence of HCVpp Fusion with Liposomes*—Our fusion assays clearly show that HCVpp membrane fusion is pH-dependent. A pH optimum of 5.0 had been reported for the cell-cell fusion activity of the HCV glycoproteins (52). However, this study was based upon chimeric HCV/vesicular stomatitis virus glycoproteins, exhibiting the ectodomains of HCV E1 and E2, and the transmembrane domain of the G protein of vesicular stomatitis virus. To determine whether any pH optimum could be observed for the fusion activation of unmodified E1/E2 displayed on HCVpp, we mixed $R_{18}$-labeled liposomes with unlabeled pseudoparticles and followed the lipid mixing kinetics over a broad pH range. We analyzed the final extent of lipid mixing (Fig. 5*A*, *open squares*), and the initial rates of lipid mixing were measured as the tangent drawn to the steepest part of the fusion curve (Fig. 5*A*, *closed squares*), obtained at a given pH, for three separate batches of pseudoparticles and liposomes. HCVpp lipid mixing occurred over a broad pH range from 4.5–6.3, with optimal rate and extent of lipid mixing at about pH 5.5 and a fusion threshold around pH 6.3 (Fig. 5*A*). These features for HCV are in line with those reported for other flaviviruses such as West Nile, Japanese encephalitis, dengue, and TBE viruses, which also display a broad pH range for fusion (see "Discussion"). In contrast, HApp exhibited a much narrower pH range for optimal fusion (pH 4.5 to 5.0; Fig. 5*B*), which is consistent with previously reported data for influenza virus fusion (50). Overall, the parallel behavior of rates and extent of fusion with pH observed for HCVpp is reminiscent of what is observed for several viral genders (Flaviviruses (29, 34), Alphaviruses (35, 37), Orthomyxoviruses (50), and Rhabdoviruses (53)).

We also studied the effect of pre-exposing pseudoparticles to low pH on HCVpp-mediated lipid mixing. HCVpp were preincubated at pH 5 in the absence of target liposomes, for the indicated times. Most interestingly, low pH pre-exposure led to a progressive inactivation of HCVpp fusogenic capacities; indeed both initial rate (Fig. 5*C*, *closed squares*) and final extent of lipid mixing (Fig. 5*C*, *open squares*) are reduced by half after only a 30-s preincubation. Note, however, that HCVpp lipid mixing still gives measurable values even after a 10-min preincubation. A similar behavior was observed for HApp on the same time scale (Fig. 5*C*, *circles*; for reasons of clarity, only two time points are indicated). This pH inactivation process implies that some HCVpp become rapidly fusion-incompetent during the fusion reaction and suggests that low pH-induced inactivation competes with low pH-induced fusion during HCVpp fusion.

*Cholesterol Facilitates the Fusion Process between HCVpp and Liposomes*—To analyze the role of cholesterol (chol) in pH-induced HCVpp-mediated lipid mixing, $R_{18}$ was incorporated into liposomes consisting of PC and chol (PC:chol; 70:30 mol %). Cholesterol at 30 mol % was chosen as an average amount, because this sterol represents 10–50 mol % of lipids in the membranes of cells, depending on their anatomical and cellular location (54). Fig. 6 shows representative curves obtained for 10 experiments made on different batches of pseudoparticles and liposomes. We observe that the addition of chol to liposomes induced a shift, for unknown reasons, in the fluorescence base line observed with all pseudoparticles and pH tested (Fig. 6, *A*, *C*, and *E* for HCVpp, HApp, and no env pp, respectively, and data not shown). However, we clearly detected higher fusion levels for HCVpp and HApp with PC, as well as PC:chol liposomes after lowering the pH significantly above the base line (Fig. 6, *A* and *B*). To tentatively correct for this unspecific base-line elevation, we subtracted for a given liposome preparation the background signal obtained at pH 7.4 from the signal obtained at pH 5.0, assuming that this nonspecific phenomenon is not pH-dependent. The corrected fusion curves are presented in Fig. 6, *B, D*, and *F* for HCVpp, HApp, and no env pp, respectively. Fig. 6*B* shows that cholesterol enhanced HCVpp-mediated lipid mixing at pH 5.0. The initial rate of HCVpp fusion was enhanced by ~20% (as visualized by *arrows* at the steepest part of the fusion kinetics), and the final extent was elevated by ~15% in the presence of cholesterol (Fig. 6*B*). These experimental observations were completed by a statistical analysis of the initial rates and final extent of lipid mixing of 10 experiments in the absence and presence of cholesterol (rank test). For HCVpp, both the initial rates and extents of lipid mixing were significantly higher in the presence than in the absence of cholesterol ($p < 0.0005$).

By contrast HApp-mediated fusion displayed similar rates and extents of fusion in the absence or presence of chol in target liposomes (Fig. 6*D*), indicating that cholesterol has no specific enhancing effect on

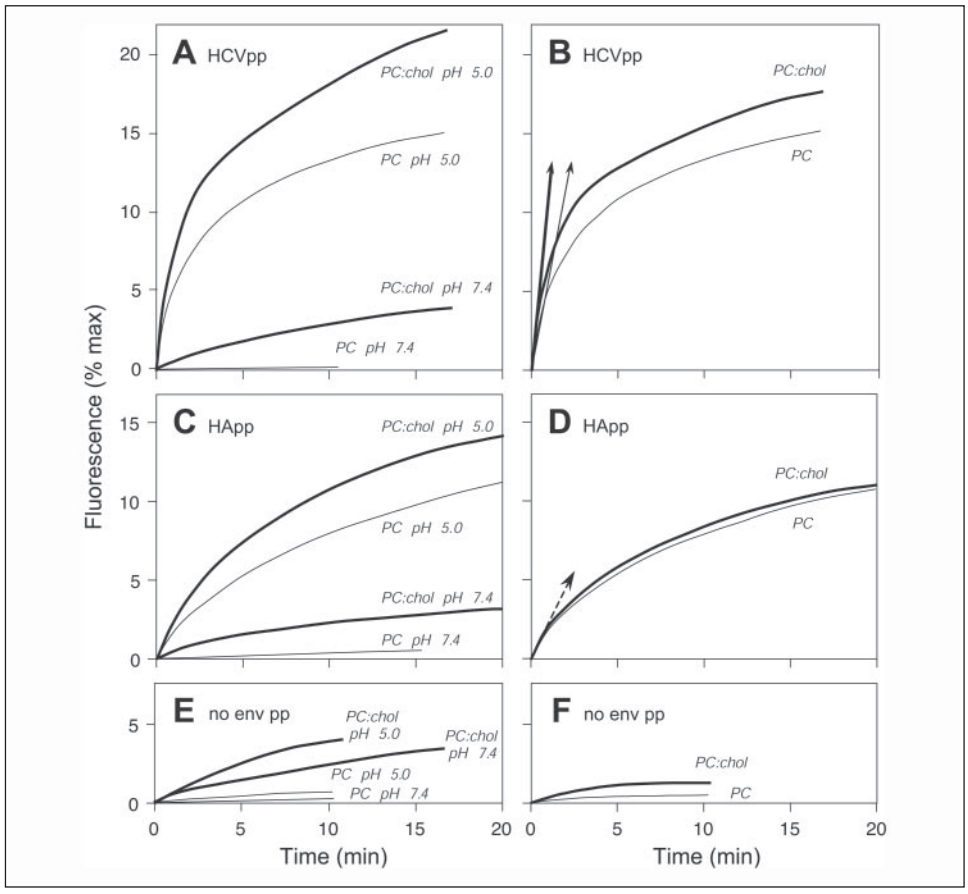


FIGURE 6. **Facilitating effect of cholesterol on HCVpp-mediated lipid mixing.** Lipid mixing as a function of time was recorded using $R_{18}$-labeled liposomes as described in Fig. 1*D*. *A,* HCVpp; *C,* HApp; and *E,* no env pp. Liposomes were composed of pure PC (*thin line*) or PC:chol (70:30 mol %; *thick line*), and the fusion kinetics were recorded at the indicated pH 5.0 or 7.4. *B,* HCVpp; *D,* HApp; and *F,* no env pp. The PC fusion curve corresponds to the substraction between curves recorded for PC at pH 5.0 and PC at pH 7.4, from *A, C,* and *E,* respectively. The PC:chol fusion curve corresponds to the substraction between curves *PC:chol pH 5.0* and *PC:chol pH 7.4* in *A, C,* and *E,* respectively. Curves are representative of those obtained in 10 separate experiments. Cholesterol has a significant effect on initial rates and final extent of lipid mixing ($p < 0.0005$, Student's *t* test statistic). *Arrows* indicate the initial rate of lipid mixing, taken as the tangent to the steepest part of the fusion curve.

hemagglutinin-induced fusion, as previously reported (55). Accordingly, based upon the rank test analysis, there was no significant difference for HApp between these parameters in the presence or absence of cholesterol.

A negligible amount of fusion was observed for no env pp with either liposome preparation after base-line correction (Fig. 6*F*). This clearly validates the no env pp as a negative control for fusion for any lipid composition tested.

Taken together, these results therefore point toward a cholesterol-facilitated mechanism for HCVpp membrane fusion, consistent with the behavior of other Flaviviruses, where cholesterol has been shown to enhance fusion (reviewed in Ref. 55).

## DISCUSSION

Various findings suggest that HCV enters cells in a pH-dependent fashion, eventually leading to the fusion of viral and cellular membranes (12–16). Here we have set up an *in vitro* fusion system, with which we demonstrate that HCVpp, a model system for the investigation of the functions of the HCV glycoproteins, mediate fusion with liposomes, including the merging of both membrane leaflets and the contents mixing of pseudoparticle and liposome. HCVpp-mediated fusion was induced by low pH, with a threshold of 6.3 and an optimum at about pH 5.5, was temperature-dependent, and did not require any protein or receptor at the surface of the target liposomes.

Our fusion studies were based on well established fusion assays and compared with the strictly pH-dependent influenza HA. HA-mediated fusion with model or cellular membranes is well documented (reviewed in Ref. 56). Consistent with reported results, we found fusion of HApp to be fast and strictly pH-dependent with a narrow pH optimum around pH 5.0. Although liposomes composed of pure PC do not constitute the most efficient target membranes for influenza virus fusion (50, 57), the fusion observed with our HApp was comparable both in terms of initial rate and extent to that observed for the wild type virus with PC liposomes (58). Control pseudoparticles devoid of any glycoprotein (no env pp) or bearing the glycoprotein of the amphotropic murine leukemia virus MLV (MLVpp), described as a receptor-dependent pH-independent fusion protein (51), confirmed that fusion in our assays was dependent on the presence of pH-dependent glycoproteins. In combination with the flexibility of pseudoparticle production, which allows incorporation of probes and genetic and protein markers as well as the analysis of various wild type and mutant glycoproteins, these fusion assays are therefore a valuable tool to separate the roles of the HCV glycoproteins E1 and E2 in the fusion process. On a more general basis, this system could be used for analyzing the fusion process mediated by any glycoprotein.

In our studies HCV E1/E2 pseudotyped particles mediated lipid mixing with liposomes of similar size (100 nm) and also of larger ones (400 nm) (data not shown). Optimal lipid mixing was observed at pH 5.5, both in terms of initial rate and final extent of the reaction (Fig. 5*A*). However, HCVpp fusion could proceed in a broad pH range with a threshold at pH 6.3, which is consistent with the fusion behavior of other Flaviviruses. Indeed, the West Nile (34), Japanese encephalitis (59), dengue (31), and TBEV (29) fuse over a broad pH range and even at almost neutral pH. In contrast, fusion observed for Alphaviruses such as Semliki Forest (SFV) and Sindbis viruses occurs at a more acidic pH in a narrow range, with a threshold at pH 6.0–6.2 (35, 37). Therefore, it is reasonable to think that after endocytosis into host cells, HCV fusion occurs in an early endosomal compartment after mild acidification.

Most interestingly, HCVpp were found to be inactivated at low pH in the absence of target membranes. Together with a potentially lower

fusogenic capacity of HCV E1E2 than that of HA (likely related to lower infectious titers), this might account for the consistently observed lower fusion extents for HCVpp when compared with HApp. However, this pH inactivation behavior is reminiscent of that reported for several virus genders, although differences were observed in the time scale of the reaction. Indeed HCVpp fusion became negligible only after low pH exposure longer than 10 min. This is notably not the case for TBEV (29), Alphaviruses such as SFV (60), and Sindbis (37), for example, where a complete fusion inactivation is achieved within the 1st min of preincubation at low pH. This points to a relative resistance of HCV to low pH in the absence of target membranes.

In our system, HCVpp-mediated fusion did not require the presence of a protein or carbohydrate receptor in the target membrane, as fusion proceeded with pure lipid vesicles. For other low pH-dependent viruses, such as the Orthomyxoviruses, Togaviruses, Flaviviruses, Rhabdoviruses, Bunyaviruses, Arenaviruses, and most probably Filoviruses, the presence of a receptor is not essential for low pH-dependent fusion, and fusion can proceed with protein-free liposomes (for a review see Ref. 56). This is in striking contrast to what is observed for pH-independent viruses such as some Retroviruses, Paramyxoviruses, Herpesviruses, Coronaviruses, and Poxviruses. Indeed, expression of their fusion capacity relies strictly on receptor-induced conformational changes in their fusion proteins (56). Consistently, we did not observe fusion in our protein-free liposomal system for MLVpp. It therefore appears to be a common theme among low pH-dependent viruses that receptor binding is not mechanistically required for membrane fusion, whereas the application of an acidic pH is a clear trigger for the fusion of these viruses. Thus, receptor binding likely induces only minor conformational rearrangements in E1 and/or E2, which are dispensable in an *in vitro* context. Alternatively, receptor-mediated conformational rearrangements in the HCV glycoproteins, acting *in vivo* as a priming process for the pH trigger, can be overcome in our *in vitro* system by low pH application. In this respect it is interesting to note that HCVpp display lower extent and slower kinetics of fusion when compared with HApp, as already noted above. Whether this reflects the fact that HCVpp fuse only suboptimally in the absence of their receptor(s) or whether this difference in fusion properties reflects the natural difference in infectious titer between the two different types of pseudoparticles remains unclear at the moment.

The influence of lipid composition on the fusion of Flaviviruses and Alphaviruses has been widely studied, in particular the influence of cholesterol and sphingolipids. SFV and Sindbis virus fusion depends strictly on the presence of cholesterol and sphingolipids in the target membrane (55, 61). By contrast the overall fusion of TBEV with liposomes is only facilitated by the presence of cholesterol in target membranes (29), is minimally affected by cholesterol depletion from cell membranes, and sphingolipids have only a weak fusion-facilitating effect (33). The flexibility of our liposome/HCVpp system allows the precise evaluation of the influence of each lipid in the fusion reaction. We have shown here that HCVpp fusion can occur with PC liposomes (Figs. 1–5) but is significantly facilitated by cholesterol when present in the target membrane (Fig. 6, *A* and *B*). Further studies are in progress, which suggest that the 3$\beta$-OH group of the cholesterol molecule is required for an optimal effect on fusion facilitation and that sphingomyelin present in target liposomes may also have a facilitating role on HCVpp fusion. Lipids can influence the fusion reaction at several stages and through different mechanisms (55) as follows. (i) They can play a role at the initial binding step, as is the case for cholesterol on SFV E1. (ii) They can locally induce membrane inhomogeneities in the target membrane or induce rafts, which are cholesterol- and sphingolipid-enriched microdomains and constitute a dynamic platform for the entry of several viruses (62). (iii) They can locally create a microenvironment of specific curvature that could influence the early interactions of a fusion protein or a fusion peptide with the target membrane (63), eventually influencing the structure and membrane orientation of the protein domain and ultimately the whole fusion process (64, 65). In particular, cholesterol is able to locally induce negative membrane curvature (66). Further studies on HCVpp cell entry could determine which of these mechanisms is modulated by cholesterol and sphingomyelin.

In conclusion this study is the first molecular investigation of the membrane fusion features of the HCV glycoproteins E1 and E2 and paves the way for a more comprehensive analysis of the fusion characteristics of HCV, as well as other pH-dependent viruses. The concept of targeting fusion as a weapon against pathogenic enveloped viruses has recently emerged, with interesting therapeutic developments in particular against HIV (reviewed in Ref. 67). Future design of small molecule fusion inhibitors requires simple and functional assay systems to allow us to gain a better molecular understanding of how fusion glycoproteins bring about fusion. The description and validation of the fusion assay system we have presented here will clearly help us to progress in that direction.

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