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Hepatitis C Virus Envelope Glycoprotein E1 Originates in the Endoplasmic Reticulum and Requires Cytoplasmic Processing for Presentation by Class I MHC Molecules

Mark Selby,* Ann Erickson,* Christine Dong,* Stewart Cooper,† Peter Parham,† Michael Houghton,* and Christopher M. Walker2*

We investigated whether hepatitis C virus envelope glycoprotein E1 is transported from the endoplasmic reticulum (ER) to the cytoplasm of infected cells for class I MHC processing. Target cells expressing E1 were killed by CTL lines from a hepatitis C virus-infected chimpanzee, and synthetic peptides were used to define an epitope (amino acids 233-GNASRCWVA-241) presented by the Patr-B*1601 class I MHC molecule. An unusually high concentration (>100 nM) of this nonameric peptide was required for target cell lysis, but this could be reduced at least 1000-fold by replacing the asparagine at amino acid position 234 (Asn234) with aspartic acid (Asp), the anticipated anchor residue for NH2-terminal peptide binding to Patr-B*1601. Conspicuously, position 234 is part of an N-glycosylation motif (Asn-Xaa-Ser/Thr), suggesting that the Asn234 to Asp substitution might occur naturally within the cell due to deglycosylation/deamidation of this amino acid by the cytosolic enzyme N-glycanase. In support of this model, we demonstrate that presentation of the epitope depended on 1) cotranslational synthesis of E1 in the ER, 2) glycosylation of the E1 molecule, and 3) a functional TAP transporter to shuttle peptide from the cytosolic to ER compartment. These results indicate for the first time that during infection of the host, viral envelope glycoproteins originating in the ER are processed in the cytoplasm for class I MHC presentation. That a posttranslational change in amino acid sequence from Asn to Asp alters the repertoire of peptides presented to CD8+ CTL has implications for the design of antiviral vaccines. The Journal of Immunology, 1999, 162: 669–676.

Epitopes presented by class I MHC molecules are generated in a multistep process that begins with attachment of ubiquitin (Ub)3 to cytoplasmic or nuclear proteins that are then targeted to the 26S proteasome for degradation into peptides. Translocation of peptides from the cytoplasm into the endoplasmic reticulum (ER) is mediated by TAP, a heterodimer encoded by TAP-1 and TAP-2 genes in the MHC (1). Class I MHC molecules in the ER are closely associated with TAP and various chaperones that facilitate loading of antigenic peptides (2). A stable complex consisting of a class I molecule, β2m, and a peptide of about 8–10 amino acids, is transported to the cell surface via the exocytic pathway for surveillance by class I MHC-associated peptides displayed by a cell. CD8+ CTL also recognize transmembrane glycoproteins, including viral envelope proteins, tumor Ags, and certain alloantigens, that are cotranslationally synthesized in the ER on membrane-bound ribosomes (4).

Processing pathways for transmembrane glycoproteins that are not normally present in the cytoplasm remain undefined. Under some circumstances processing can occur in the ER rather than the cytoplasm. For instance, signal peptidase (5–8) or other unidentified proteases (6, 9) can generate peptides that bind directly to class I MHC molecules in the ER, thus bypassing the cytoplasmic Ub/proteasome/TAP pathway. Most epitopes from transmembrane glycoproteins are TAP dependent, however, suggesting that at least some processing events occur in the cytoplasm.

At least two different mechanisms could account for cytoplasmic processing of proteins that normally localize to the ER. In the first mechanism, transmembrane glycoproteins may occasionally be synthesized on free cytoplasmic rather than membrane-bound ribosomes, and thus targeted for ubiquitination and destruction by the proteasome. Studies of an HLA-B35-restricted epitope from the HIV-1 gp120 envelope indicate that this pathway is functional in virus-infected cells (9, 10). The gp120 envelope contains a number of signal motifs for Asn-linked glycosylation (Asn–Xaa–Ser/Thr, where Xaa is any amino acid except Pro), and all are known to be modified by glycans during synthesis of the polyprotein in the ER (11). One of these Asn residues is contained in the epitope, but surprisingly was not glycosylated before presentation by the HLA-B35 molecules (10). This result suggests that gp120 polyprotein produced in the cytoplasm because of an error in translation (12–14) or possibly signal peptide-mediated targeting to the ER (9, 10) was the substrate for class I MHC processing.

A second mechanism appears to involve export of defective transmembrane glycoproteins from the ER to the cytoplasm for proteasome-mediated destruction (15–20). This pathway is supported by recent studies of class I MHC-restricted epitopes in the influenza virus nucleoprotein (21) and the cellular glycoprotein tyrosinase that is expressed in melanocytes (22, 23). In the case of
tyrosinase, it was demonstrated that efficient presentation of one TAP-dependent epitope by HLA-A2 required posttranslational conversion of an encoded Asn residue to Asp (22). This required cotranslational glycosylation of tyrosinase in the ER and subsequent translocation of the Ag to the cytoplasm where it was deglycosylated (23), probably by peptide:N-glycanase (PNGase) (24, 25). Removal of glycans by this cytoplasmic enzyme causes a coding change from Asn to Asp by a process of deamidation, and it is this modified form of tyrosinase that is processed by the Ub/proteasome/Tap pathway. Whether this processing pathway applies generally to other glycoproteins is not yet known.

In this study we demonstrate for the first time that the ER to cytosol processing pathway is not restricted to cellular glycoproteins such as tyrosinase, but is also operative for viral glycoproteins produced in infected cells. Class I MHC-restricted CTL specific for envelope glycoproteins E1 and E2 of hepatitis C virus (HCV) are present in the liver and peripheral blood of infected humans (26–31) and chimpanzees (32–34). For at least one TAP-dependent E1 epitope, cotranslational glycosylation was strictly required for Ag presentation, presumably because an associated Asn to Asp substitution facilitated peptide binding to class I MHC molecules. This posttranslational modification also appeared to influence the repertoire of HCV-specific CD8+ CTL in an infected chimpanzee, and therefore has implications for vaccine design.

**Materials and Methods**

**CTL lines**

Chimpanzee Ross (CH-503) developed a persistent infection after experimental challenge with 100 chimpanzee infectious doses of the HCV-1-910 (genotype 1a) virus stock. Plasma viremia has been consistently detected through 5 yr of follow-up study (32). The class I MHC phenotype of this chimpanzee is Patr-A*0401, A1*0101, B1*0106, B7*0101, C0*0501, C0*0601 (33). HCV-specific CTL lines were generated from the liver of this animal as described previously (32). CTL line 503/11.3 is specific for a Patr-A*0401-restricted epitope spanning amino acids 588–596 (KHP-DATYSR) of the HCV E2 glycoprotein (32, 33). CTL lines 503/13.4 and 503/10D recognize Patr-B*1601-restricted epitopes in E1 and nonstructural 3 (NS3) proteins of HCV-1, respectively (32, 33). CTL lines specific for the Patr-B*1601-restricted E1 epitope were also generated by incubation of 4 × 10^6 Fc-coated-Hypo-separate PBMC in RPMI 1640 culture medium containing 10% FCS, 20 (20 μl), 10% FCS, 0.5% FCS, and synthetic HCV E1 peptides at 10 μg/ml as previously described (30). After 7 days, lymphocytes were washed once and restimulated with 1 × 10^6 autologous irradiated (3000 rad) PBMC in culture medium supplemented with IL-2 and synthetic HCV peptide. After a further 7 days, CD8+ T cells were enriched from the cultures using immunomagnetic beads (32) and were tested for HCV-specific lytic activity as described below.

**Recombinant vaccinia viruses**

Vaccinia viruses Vwt, VC/E1, and VE1/2 were described previously (30). Materials and Methods. Recombinant vaccinia virus VV-ICP47 expressing the ICP47 protein of herpes simplex virus type 1 (35) was kindly provided by Dr. Barry Rouse, University of Tennessee (Knoxville, TN).

**Synthetic peptides**

HCV-1 peptides were synthesized by Chiron Mimotopes (Clayton, Australia) or Research Genetics (Huntsville, AL) using Fmoc solid phase methods. All peptides had free amino and carboxyl termini.

**Cytotoxicity assays**

A B lymphoblastoid target cell line was established from the peripheral blood of chimpanzee CH-503 by transformation with supernatant from the marmoset cell line B95-8 as previously described (32). Target cells were infected with recombinant vaccinia viruses expressing HCV-1 Ags at a multiplicity of infection (moi) of 10. In experiments involving VV-ICP47, targets were first infected for 1 h with this virus or Vwt at an moi of 15 and then superinfected with recombinant viruses expressing the HCV E1 glycoprotein for an additional 2 h at a moi of 5. All vaccinia virus-infected targets were washed and cultured overnight before labeling with 51Cr. In some experiments target cells were sensitized with synthetic HCV-1 peptides during 51Cr labeling. After three washes, 5 × 10^3 target cells were added to duplicate wells of a 96-well plate with varying numbers of CD8+ effector cells. Target cells cultured alone in medium or detergent (1% Nonidet P-40) provided the minimum and maximum release values, respectively. Plates were incubated for 4 h at 37°C, then 50 μl of culture supernatant was harvested onto 96-well Lumaplates (Packard, Downers Grove, IL), dried overnight, and counted in a Wallac 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD). The percent specific 51Cr release was calculated using the formula: [(test release – minimum release)/(maximum release – minimum release)] × 100.

**Tunicamycin treatment of target cells**

Tunicamycin was added at a concentration of 10 μg/ml of culture medium 1 h before infection with recombinant vaccinia viruses, and targets were maintained in the presence of drug during assays for Ag expression or susceptibility to CTL lysis. The m.w. of HCV E1 and E2 Ags in drug-treated and untreated cells were assessed by Western blot analysis. Briefly, cell lysates were pelleted and resuspended in Laemmli sample buffer. The lysates were sheared with an insulin hypodermic syringe, and the debris was pelleted. Supernatants were loaded onto 14% SDS gels for electrophoresis and then transferred to nitrocellulose in 20% methanol/electrophoresis buffer. Blots were blocked and then incubated with the appropriate mAb diluted 1/1000 (anti-E1:3D5/C3; anti-E2:3E5–1) for 1 h at room temperature. After washing, enhanced chemiluminescence reagents (Amer sham, Arlington Heights, IL) were added, and the blots were exposed to film.

**Results**

A CD8+ CTL line designated 503/13.4 was established from liver tissue biopsied 28 wk after infection of chimpanzee Ross with the genotype 1a HCV-1 isolate (32). Autologous target cells infected...
with recombinant vaccinia viruses expressing the HCV-1 E1 envelope glycoprotein were lysed by this CTL line (Fig. 1A). The epitope recognized by CTL line 503/13.4 was identified by pulsing target cells with a series of overlapping decameric peptides that spanned the HCV-1 E1-coding sequence. Only one peptide (amino acids 232-EGNASRCWVA-241) sensitized the target cells (data not shown), and further truncation of NH2- and COOH-terminal amino acids revealed that a nine-amino acid peptide (amino acid 233-GNASRCWVA-241) is the minimum optimal epitope (Fig. 1B). This peptide was designated 58N, where the superscript indicates the amino acid residue at position 234 in single letter code. A striking feature to emerge from these studies was the high concentration of peptide 58N required to sensitize target cells for lysis; a peptide concentration exceeding 200 nM was needed to match the levels of killing detected against targets infected with VE1/2. Epitope 58N was previously shown to be presented by the class I MHC allotype Patr-B*1601 (33). Notably, a second Patr-B*1601-restricted CTL line, designated 503/10D, was derived from the liver of the same chimpanzee. It recognized a nonameric epitope in the NS3 protein of HCV-1, and efficient lysis of target cells was achieved with peptide concentrations as low as 0.1 nM (Fig. 2A) (36). Comparison of the E1 and NS3 epitopes revealed a difference at amino acid position 2 that is probably the dominant NH2-terminal anchor residue for peptide binding to Patr-B*1601 (Fig. 2A). We hypothesized that the Asp at position 2 of the NS3 epitope facilitated efficient presentation by Patr-B*1601, and that the E1 epitope would be better presented if Asn234 was replaced by Asp. As shown in Fig. 2B, the substituted peptide (designated 58D; GDASRCWVA) sensitized target cells for lysis over a wide range of concentrations. Half-maximal lysis of targets occurred at a peptide concentration of about 0.1 nM, an amount comparable to that required for the NS3 epitope restricted by Patr-B*1601 (36). By contrast, peptide 58N failed to sensitize targets at any of the concentrations tested in this experiment. To assess whether CTL populations against either 58N or 58D sequences dominated in vivo, PBMC obtained from CH-503 at 263 wk postinfection were stimulated twice at weekly intervals with each individual peptide. CTL activity was assessed after an additional week of culture. CD8+ T cells enriched from all six replicate PBMC cultures stimulated with peptide 58N had low or no detectable lytic activity against targets sensitized with the homologous peptide compared with that against control targets (Fig. 3A). In contrast, high levels of 58D-specific lytic activity were detected in all six cultures stimulated with this Asp-substituted peptide (Fig. 3B). One representative cell line (B58D.3) efficiently killed targets infected with VE1/2 (Fig. 3C). Moreover, when target cells were treated with peptides 58N and 58D, only the latter was recognized over a concentration range from 10 – 0.001 nM (Fig. 3D). Thus, CTL against the E1 epitope were still present in chimpanzee Ross after 5 yr of persistent infection and showed the same preference for the Asp-substituted peptide as the intrahepatic CTL line 503/13.4 established during the acute phase of hepatitis C. This result strongly suggests that the dominant form of this epitope eliciting
CD8<sup>+</sup> CTL in the infected host contains an Asp rather than an Asn residue at amino acid position 234.

Notably, Asn 234 is part of an Asn-Xaa-Ser/Thr consensus glycosylation motif in the E1 protein (Fig. 2A). Modification of this residue by glycosylation during cotranslational synthesis of E1 might therefore influence presentation of the epitope. To address this question, target cells were infected with vaccinia viruses expressing no HCV Ag (Vwt; ×), E1 and E2 (VE1/2; ▲), and core and E1 (VC/E1; ●). The percent specific lysis by CTL line B58<sup>0.3</sup> was assessed in a 4-h assay. D. Target cells were sensitized with the indicated concentration of peptide 58<sup>N</sup> (●) or 58<sup>D</sup> (▲) and cocultured with CTL line B58<sup>0.3</sup> at an E:T cell ratio of 20:1 for 4 h.

If cotranslational glycosylation of the Asn<sup>234</sup> residue is important for presentation of the E1 epitope by Patr-B<sup>1601</sup>, then limiting Ag expression to the cytoplasm of target cells by removal of the signal peptide sequence should prevent target cell recognition by CD8<sup>+</sup> CTL. Studies with a panel of recombinant vaccinia viruses expressing modified E1 Ags (Fig. 6A) support this contention. Virus VE1<sub>s</sub> expressed full-length E1, which consists of an

FIGURE 3. Characterization of 58<sup>D</sup>- and 58<sup>N</sup>-specific CTL lines derived from peripheral blood. PBMC were stimulated twice with peptide 58<sup>N</sup> (A) or peptide 58<sup>D</sup> (B) and enriched CD8<sup>+</sup> T cells were tested for lysis of autologous, 51Cr-labeled LCL that were untreated or sensitized with a 10-nM concentration of homologous (i.e., 58<sup>N</sup> or 58<sup>D</sup>) peptide or irrelevant control peptide 162A. C. Autologous target cells were uninfected (■) or were infected with vaccinia viruses expressing no HCV Ag (Vwt; ×), E1 and E2 (VE1/2; ▲), and core and E1 (VC/E1; ●). The percent specific lysis by CTL line B58<sup>0.3</sup> was assessed in a 4-h assay. D. Target cells were sensitized with the indicated concentration of peptide 58<sup>N</sup> (F) or 58<sup>D</sup> (Œ) and cocultured with CTL line B58<sup>0.3</sup> at an E:T cell ratio of 20:1 for 4 h.

FIGURE 4. Western blot of tunicamycin-treated, VVE1/E2-infected cells. Lysates of LCL infected with a control VV (Vwt; lanes 1 and 2) or VE1/2 (lanes 3 and 4) at an moi of 10 were loaded onto a 14% gel for electrophoresis. After transfer to nitrocellulose, blots were reacted with an HCV E1-specific mAb. Cells in lanes 2 and 4 were infected in the presence of tunicamycin at a concentration of 10 µg/ml of culture medium.
initiation codon followed by the natural signal peptide (amino acids 171–191) and mature envelope glycoprotein (amino acids 192–383; Fig. 6A). Target cells infected with this virus or pulsed with peptide 58D were killed at equivalent levels, confirming that the normal synthetic pathway for E1 in the infected cell, which involves cotranslational glycosylation in the ER, leads to efficient presentation of the Patr-B*1601-restricted epitope (Fig. 6B). As predicted, presentation of this epitope was prevented if glycosylation of the Asn234 residue was blocked by restricting E1 expression to the cytoplasm. Target cells infected with VE1s−, which expresses E1 without its natural leader peptide (Fig. 6A), were not susceptible to CTL lysis (Fig. 6B). We reasoned that cytoplasmic expression of E1 would be sufficient for class I MHC presentation of this epitope if an Asn to Asp mutation was introduced at position 234, bypassing any normal requirement for glycosylation/deglycosylation of this residue to create an optimal anchor for Patr-B*1601 binding. A virus designated VE1s−D234 that carries this mutation in E1 (Fig. 6A) sensitized target cells (Fig. 6B). Indeed, equivalent levels of killing against target cells infected with VE1s+ and VE1s−D234 were observed at all three E:T cell ratios, indicating that an N234 to D substitution completely replaced the requirement for a functional signal peptide in presentation of this epitope.

Processing of the E1 glycoprotein in the cytoplasm after retrograde transport from the ER should require a functioning TAP apparatus to reintroduce the deglycosylated epitope into the ER for class I MHC presentation. To investigate this issue, target cells were infected with a recombinant vaccinia virus expressing the HSV-1 ICP47 protein.

FIGURE 5. Lysis of tunicamycin-treated target cells by HCV-envelope specific CTL lines. Autologous LCL were untreated (■) or were infected with control Vwt (●) or VE1/2 in the presence (▲) or the absence (▼) of tunicamycin at a concentration of 10 μg/ml of culture medium. Targets were then assessed for lysis by E1-specific CTL line 13.4 (A) or E2-specific line 11.3 (B) at the indicated E:T cell ratios in a 4-h assay.

FIGURE 6. A. Recombinant vaccinia viruses expressing HCV E1 envelope proteins. The amino acid at position 234 is shown in brackets in single letter code. B. Cytolysis of target cells expressing HCV E1 Ags. Autologous LCL were untreated (▲), sensitized with peptide 58D (●), or infected with vaccinia viruses expressing no HCV Ag (Vwt; ✷), E1 and E2 (VE1/2; ■), full-length E1 with signal peptide sequence (VE1s+; ●), E1 lacking signal sequence (VE1s−; ●), and E1 lacking signal sequence with an N234→D substitution (VE1s−D234; ▼).
Fig. 7. HCV E1 epitope is TAP dependent. A. Target cells were untreated (▲), sensitized with peptide 58^D (●), or infected sequentially with Vwt/Vwt (■) or VV-ICP47/Vwt (◆) as described in Materials and Methods. Targets were also infected with VE1_{−d234} (B, ■) or VE1/2 (C, ▲) alone or after infection with Vwt (◆) or VV-ICP47 (▲). The percent specific lysis was assessed after a 4-h coculture with CTL line B58^D.3

Discussion
This study demonstrates for the first time that viral envelope glycoproteins originating in the ER are translocated to the cytoplasm for class I MHC processing, and thus appear to follow the same pathway recently described for cellular glycoproteins such as tyrosinase (22, 23). Our results indicate that the classical class I MHC pathway, which is known for efficient presentation of cytoplasmic or nuclear Ags, also processes membrane glycoproteins of intracellular pathogens or tumors that might otherwise avoid immune recognition by cotranslational synthesis and/or modification by Asn-linked glycans.

Presentation of the HCV E1 epitope by Patr-B*1601 was at least 1000-fold more efficient when the Asn234 residue encoded by HCV-1 was replaced by Asp. Modeling studies indicate that this amino acid switch is required for peptide binding to the B pocket of Patr-B*1601, and the presence of an Asp residue at position 2 of the HCV NS3 epitope (Fig. 2A) is consistent with this view. Given the poor presentation of synthetic peptide 58^D by Patr-B*1601, it seemed improbable that an epitope containing Asn234 could elicit CTL in the infected host. Indeed, expansion of CD8^T CTL from an HCV-infected human subject (26) even though peptide binding to the HLA-B35 restriction element was reportedly several orders of magnitude less efficient than that of other synthetic peptides considered immunogenic (27). An Asn234 to Asp substitution could also explain presentation of this epitope in humans, because peptide binding to HLA-B^B3501 is sometimes mediated by a position 2 Asp instead of the usually dominant Pro anchor residue (38). Posttranslational changes in primary amino acid sequence from Asn to Asp would allow a greater repertoire of epitopes to be presented (Fig. 2A), which might explain the variability of the epitope presentation among individuals with different HLA-B restriction elements. These results demonstrate that cytoplasmic synthesis of E1 encoding the optimal Asp234 residue resulted in efficient lysis of target cells, and this was prevented by prior infection with VV-ICP47 but not Vwt. Significantly, infection of the targets with VV-ICP47 before superinfection with VE1/2, which expresses full-length E1 with signal peptide, also prevented presentation of the epitope (Fig. 7C). These results demonstrate that TAP is needed for Patr-B*1601-restricted recognition of this epitope regardless of whether E1 is synthesized in the cytoplasm (VE1_{−d234}; Fig. 7B) or the ER (VE1/2; Fig. 7C).

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inhibitors also prevent presentation of the E1 epitope (C. M. Walker, unpublished observations). We postulate that deglycosylation of E1 is particularly important for immune recognition of HCV-infected cells. The most plausible mechanism is enzymatic hydrolysis of the β-asparagylucosaminy bond by PNGase; deamidation of the Asn\textsuperscript{254} residue appears to be an absolute requirement for peptide presentation by Patr-B\textsuperscript*1601. It is conceivable that for other epitopes, Asn to Asp substitutions cause more subtle alterations in immune recognition, particularly if these residuals are important for TCR recognition instead of anchoring to class I MHC molecules.

Morrison and colleagues (46) originally demonstrated that the hemagglutinin protein of influenza virus must be synthesized in an infected cell for effective recognition by CD8\textsuperscript+ T cell. Hemagglutinin synthesized in cells without a leader peptide for ER localization is not efficiently presented for CD8\textsuperscript+ T cell recognition (47). These studies raised questions about whether glycoproteins processed for class I MHC presentation originate in the cytoplasm or the ER. Although the present study indicates that this can involve retrograde transport of membrane glycoproteins from the ER to the cytosol, it is probably not the only pathway in infected cells leading to class I MHC presentation of TAP-dependent epitopes. For instance, some epitopes of gp120 contain Asn-Xaa-Ser/Thr motifs but are neither glycosylated nor deglycosylated before class I MHC presentation (10). This suggests that a small fraction of envelope proteins are aberrantly synthesized on free cytoplasmic ribosomes because of a transient shortage of signal recognition particles that chaperone nascent polypeptides and attached ribosomes to the ER membrane (10) or because of errors in translation (13). Taken together, studies of tyrosinase and transmembrane envelopes of HCV and HIV-1 suggest that glycoproteins synthesized in cells without a leader peptide for ER localization are not the exclusive mechanism of immune evasion by HCV.

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