Hepatitis C virus mutation affects proteasomal epitope processing

Ulrike Seifert,[1] Heike Liermann,[2] Vito Racanelli,[3] Anne Halenius,[4] Manfred Wiese,[5] Heiner Wedemeyer,[3] Thomas Ruppert,[1] Kay Rispeter,[6] Peter Henklein,[1] Alice Sijts,[1] Hartmut Hengel,[4] Peter-M. Kloetzel,[1] and Barbara Rehermann[2,3]

[1]Institute of Biochemistry, Charité, Humboldt University, Berlin, Germany. [2]Department of Gastroenterology, Hepatology and Endocrinology, Medizinische Hochschule Hannover (MHH), Hannover, Germany. [3]Liver Diseases Section, Digestive Diseases Branch, National Institute for Diabetes and Digestive and Kidney Diseases (NIDDK), NIH, Department of Health and Human Services (DHHS), Bethesda, Maryland, USA. [4]Robert Koch-Institute, Berlin, Germany. [5]II. Klinik für Innere Medizin, Städtisches Klinikum St. Georg, Leipzig, Germany. [6]Institute for Virology, Universitätsklinikum, Essen, Germany.

The high incidence of hepatitis C virus (HCV) persistence raises the question of how HCV interferes with host immune responses. Studying a single-source HCV outbreak, we identified an HCV mutation that impaired correct carboxyterminal cleavage of an immunodominant HLA-A2–restricted CD8 cell epitope that is frequently recognized by recovered patients. The mutation, a conservative HCV nonstructural protein 3 (NS3) tyrosine to phenylalanine substitution, was absent in 54 clones of the infectious source, but present in 15/21 (71%) HLA-A2–positive and in 11/24 (46%) HLA-A2–negative patients with chronic hepatitis C. In order to analyze whether the mutation affected the processing of the HLA-A2–restricted CD8 cell epitope, mutant and wild-type NS3 polypeptides were digested in vitro with 20S constitutive proteasomes and with immunoproteasomes. The presence of the mutation resulted in impaired carboxyterminal cleavage of the epitope. In order to analyze whether impaired epitope processing affected T cell priming in vivo, HLA-A2–transgenic mice were infected with vaccinia viruses encoding either wild-type or mutant HCV NS3. The mutant induced fewer epitope-specific, IFN-γ–producing and fewer tetramer$^+$ cells than the wild type. These data demonstrate how a conservative mutation in the flanking region of an HCV epitope impairs the induction of epitope-specific CD8$^+$ T cells and reveal a mechanism that may contribute to viral sequence evolution in infected patients.

Introduction

Hepatitis C virus (HCV) is a 9.6 kb positive-stranded RNA virus of the flavivirus family and the leading cause of chronic hepatitis worldwide. Whereas recovery from acute HCV infection has been associated with multispecific T cell responses that protect upon reexposure to the virus (1, 2), these responses are either not induced or not maintained in the large number of patients who develop chronic infection (3–5).

We have therefore asked whether HCV interferes with the induction of antigen-specific T cells. Induction of CD8$^+$ T cells depends on the generation of MHC class I ligands by the proteasome, the major cytosolic proteinase. The proteasome cleaves short peptides from longer polypeptide precursors that are then translocated into the endoplasmic reticulum and bind to newly synthesized MHC class I molecules (6). 26S proteasomes contain a 20S catalytic core, arranged as 2 heptameric outer rings with 7 α-subunits each and 2 heptameric inner rings with 7 β-subunits each. Proteasome activity is closely regulated by cytokines that are produced in viral infections (7–12). In response to IFN-γ, for example, the constitutive catalytic subunits β1, β2, and β5 are replaced by low molecular weight protein 2 (LMP2) (iβ1), LMP7 (iβ5), and multicatalytic endopeptidase complex-like–1 (MECL-1) (iβ2) to form immunoproteasomes with altered cleavage properties (13).

HCV circulates in an abundant number of quasispecies because of its high replication rate (14) and its lack of polymerase proofreading capacity. Individual HCV sequences have been described as abolishing recognition by T cell receptors (TCRs) and antibodies, thus interfering with the effector arms of the cellular (2, 15, 16) and humoral immune responses (17). In contrast, the possibility that HCV mutations affect the induction of T cell responses has not been investigated. Indirect evidence for this hypothesis stems from descriptive reports that HCV isolates from persistently infected patients often encode less immunogenic sequences than prototype peptides used for in vitro analysis (18, 19). Whether the decreased immunogenicity results from viral mutations or from infection with less immunogenic strains has not been analyzed because the sequence of the infecting virus is not known in most human studies and also because the route of infection and inoculum size differ among the studied individuals.

Having studied a cohort of patients accidentally infected by a single-source HCV with known sequence, we here demonstrate that an HCV mutation located in the flanking region of a frequently recognized HCV epitope (4, 20–25) impairs the induction of HCV-specific CD8$^+$ T cells by affecting the sophisticated proteasomal antigen-processing machinery.

Results

The tyrosine/phenylalanine mutation at residue HCV nonstructural protein 3$_{1082}$ is common in patients who develop persistent infection after a single-source outbreak of HCV. In order to study viral mutations during the natural course of HCV infection in humans, we analyzed a cohort of patients that had accidentally been infected with HCV in 1978/1979 during a single-source outbreak due to a contaminated anti–D immunoglobulin (26). Because the precise time of infection as well as the genotype and the sequence of the original infectious virus were known and identical for all patients, this cohort was

Nonstandard abbreviations used: hepatitis C virus (HCV); nonstructural protein 3 (NS3); phenylalanine (F); tyrosine (Y).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **114**:250–259 (2004). doi:10.1172/JCI200420985.

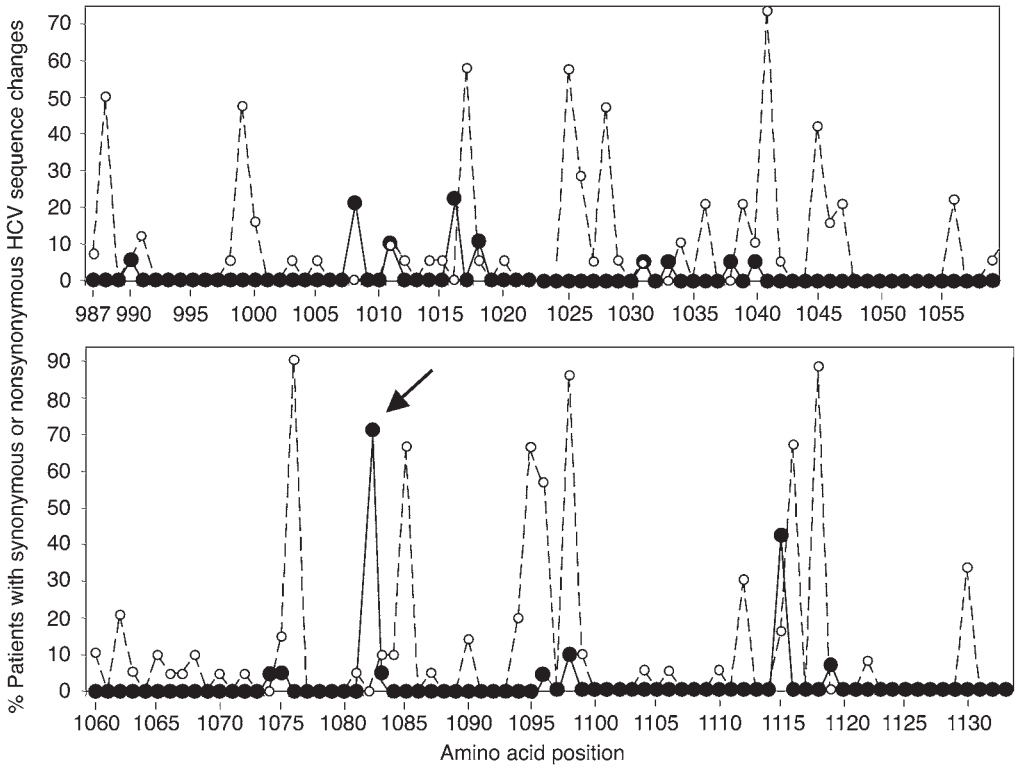

Figure 1
Prevalence of synonymous (open circles) and nonsynonymous (filled circles) HCV mutations within the HCV $NS3_{987–1133}$ sequence isolated from HLA-A2 positive, persistently infected HCV patients 18 years after a single-source outbreak of hepatitis C. The arrow indicates the amino acid position with the highest rate of nonsynonymous to synonymous mutations.

suitable for studying candidate mechanisms of viral persistence. As previously described for this and other cohorts, the strength of the cellular immune response correlated with the outcome of infection, and HCV nonstructural protein 3 (NS3) peptides were among the most frequently recognized (3, 5, 20, 27, 28).

When we compared the HCV $NS3_{987–1133}$ sequence of the infectious source with sequences that we isolated from the sera of persistently infected, HLA-A2–positive patients 18 years after the single-source outbreak, we found the highest ratio of nonsynonymous to synonymous HCV mutations at amino acid position $NS3_{1082}$ (Figure 1). Position $NS3_{1082}$ was located directly adjacent to the carboxyterminus of an HLA-A2–restricted $CD8^+$ T cell epitope recognized by recovered patients of this (20) and other cohorts (4, 20–25). Whereas 54 molecular clones from 3 independent PCRs of the infectious source encoded a tyrosine (Y) in position $NS3_{1082}$, serum isolates from 15 of 21 (71%) HLA-A2–positive, persistently infected patients encoded a phenylalanine (F) at position $NS3_{1082}$ (Table 1). Because the Y/F mutant was also observed in a substantial number of HLA-A2–negative, persistently infected patients (11 of 24 [46%]; $P = 0.07$; data not shown), the epidemiological data alone did not indicate whether HLA-A2–restricted T cell selection pressure could have contributed to the viral sequence evolution in this patient cohort. We therefore decided to analyze the molecular and immunological effects of the Y/F mutation in vitro by studying proteasomal processing of antigen and in vivo by studying its effect on T cell priming in an HLA-transgenic mouse model.

The Y/F mutation at residue $N3_{1082}$ impairs carboxyterminal processing of the $NS3_{1073–1081}$ epitope. Most MHC class I–restricted peptides are liberated from antigenic precursor sequences by the 20S core particle of the proteasome. To analyze whether the $NS3_{1082}$ Y/F substitution affected proteasomal processing of the $NS3_{1073–1081}$ epitope, we built on our previous demonstration that proteasome-dependent in vitro processing of epitope-harboring polypeptides reflects the in vivo situation with high fidelity (10, 12, 29, 30) and compared proteasome-dependent in vitro processing of the NS3 wild-type polypeptide, designated $NS3(Wt)_{1062–1095}$, with processing of the NS3 mutant polypeptide, designated $NS3(Mut)_{1062–1095}$. For this purpose, immunoproteasomes were purified from IFN-γ–stimulated HepG2 human hepatoma cells and from murine mouse embryonal cells–217 (MEC-217) transfected with the immunoproteasome subunits LMP2, LMP7 and MECL-1 (10). Constitutive proteasomes were purified from unstimulated HepG2 cells and from murine MEC-18 cells.

Synthetic $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptides (Figure 2a) were then incubated with 20S immunoproteasomes. The digestion products were separated and analyzed by reverse phase–HPLC and mass spectrometry. $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptide substrates were digested with the same kinetics with nearly 40–50% turn-over of each substrate within 4–8 hours (Figure 2B). Because longer digestion times resulted in secondary cleavage of processing intermediates, 4-hour and 8-hour digestion times were considered optimal for further biochemical analyses. Processing with constitutive proteasomes yielded the same qualitative results but lower amounts of digest product than processing with immunoproteasomes (not shown).

In contrast to the substrates, the relative abundance of the $NS3_{1073–1081}$ epitope could not be precisely determined because the epitope's 2 cysteine residues formed aggregates via disulfide bonds. These aggregates interfered with reliable identification of the $NS3_{1073–1081}$ epitope and its immediate precursors by mass spectrometry. As an indirect measure of the generation of the $NS3_{1073–1081}$ epitope, we therefore determined the relative abundance of those cleavage products that flanked its amino- and carboxyterminus. Cleavage product $NS3_{1062–1070}$, for example, flanked the amino-terminus of the $NS3_{1073–1081}$ epitope. Cleavage product

Table 1
HCV $NS3_{1062–1095}$ sequence of the infectious source and HLA-A2+ HCV-infected patients

Experiment/patient	$HCV_{NS3\ 1062–1094}$ amino acid sequence	Analyzed molecular clones/ PCR product
Infectious source		
PCR 1	VSTATQSFLATCVNGVCWTVYHGAGSKTLAGPKG	16 clones
	----------E-------	1 clone
	----------R-------	1 clone
	----------L----------	1 clone
	----------A----------	1 clone
	----------A----------	1 clone
PCR 2	----------	14 clones
	----------S--	1 clone
	----------L----------	1 clone
PCR 3	----------	12 clones
	----------A----------	2 clones
	----------R----------	1 clone
	----------T----	1 clone
	----------R----------	1 clone
HLA-A2+, persistently infected HCV patients		
Patient 1	----------	17 clones
	----------A----------	1 clone
	----------A----------	1 clone
Patient 2	----------	12 clones
	----------A----------	1 clone
	----------E--	1 clone
	----------D----	1 clone
Patient 3	----------	PCR product
Patient 4	----------	PCR product
Patient 5	----------S----------	PCR product
Patient 6	----------	PCR product
Patient 7	----------F----------	15 clones
	-----L----------F----------	1 clone
Patient 8	----------F----------	19 clones
	----------F-S----------	1 clone
Patient 9	----------F----------	12 clones
	----------FQ----------	1 clone
Patient 10	----------F----------	10 clones
Patient 11	----------F----------	14 clones
	----------S-------F----------	1 clone
	--------A----------F----------	1 clone
	-------V----------F----------	1 clone
Patient 12	----------F----------	PCR product
Patient 13	----------F----------	PCR product
Patient 14	----------F----------	PCR product
Patient 15	----------F----------	PCR product
Patient 16	----------F----------	PCR product
Patient 17	----------F----------	PCR product
Patient 18	----------F----------	PCR product
Patient 19	----------S-------F----------	PCR product
Patient 20	----------I-------F----------	PCR product
Patient 21	----------F----------	PCR product

$NS3_{1062–1070}$ was generated from both the $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptide substrates with comparable efficiency (Figure 2C). Generation of cleavage product $NS3_{1062–1070}$ was associated with the generation of the complementary cleavage product $NS3_{1071–1095}$ (Figure 2D), consistent with a proteasomal cut between amino acid positions 1070 and 1071 in both the $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptide substrates (see dotted line in Figure 2A). This proteasomal cut resulted in an amino-terminal elongation of the $NS3_{1073–1081}$ epitope by 2 amino acids.

A second cleavage product that flanked the amino-terminus of the $NS3_{1073–1081}$ epitope was peptide $NS3_{1062–1072}$. As indicated in Figure 2E, cleavage product $NS3_{1062–1072}$ was also liberated from both the $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptide substrates in comparable amounts (Figure 2E), indicating that the proteasome cut both the $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptide between amino acid positions 1072 and 1073 as indicated by the dotted line in Figure 2A. This proteasomal cut resulted in the generation of the correct amino-terminus of the $NS3_{1073–1081}$ epitope, irrespective of the presence or absence of the $NS3_{1082}$ mutation. The demonstration that the $NS3_{1073–1081}$ epitope and its amino-terminally elongated forms were generated at the same time is consistent with previous findings for other epitopes (31, 32). In cases of amino-terminally elongated peptides, the precise amino-terminus has been shown to be further defined by postproteasomal trimming by aminoexopeptidases (31, 32).

In contrast to the amino-terminus, the correct carboxyterminus of the $NS3_{1073–1081}$ epitope was generated only from the wild-type polypeptide. Figure 2 (F, G, and H) shows the relative abundance of those cleavage products whose generation defined the epitope's carboxyterminus at amino acid position $NS3_{1082}$. The generation of cleavage product $NS3_{1082–1095}$ indicated the correct processing of the epitope's carboxyterminus with a proteasomal cut between amino acid positions 1081 and 1082 (Figure 2F). Importantly, cleavage product $NS3_{1082–1095}$ was only liberated from the $NS3(Wt)_{1062–1095}$ and not from the $NS3(Mut)_{1062–1095}$ polypeptide substrate (Figure 2F). In contrast, cleavage product $NS3_{1083–1095}$ was liberated from the $NS3(Mut)_{1062–1095}$ more efficiently than from the $NS3(Wt)_{1062–1095}$ polypeptide substrate (Figure 2G). This result indicated that, in the presence of the $NS3_{1082}$ mutation, the proteasome cut between amino acid positions 1082 and 1083 rather than between amino acid positions 1081 and 1082 and thus generated

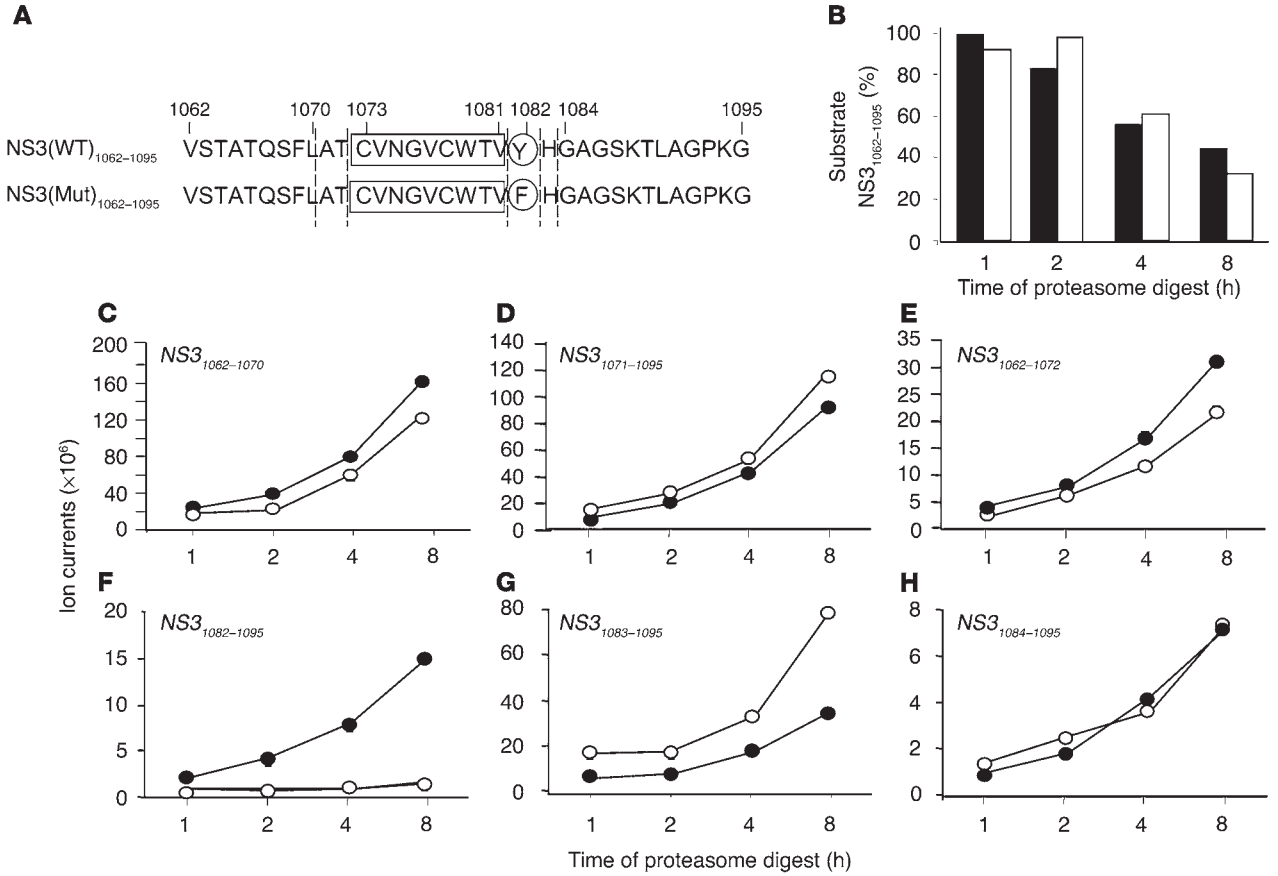

Figure 2

Digestion of NS3(Wt)$_{1062–1095}$ polypeptide and NS3(Mut)$_{1062–1095}$ polypeptide with purified 20S immunoproteasomes. (**A**) The amino acid sequences of the 34-mer NS3(Wt)$_{1062–1095}$ and NS3(Mut)$_{1062–1095}$ polypeptides are shown in single-letter code. The HLA-A2 restricted CD8$^+$ T cell epitope NS3$_{1073–1081}$ is framed, and amino acid position NS3$_{1082}$ containing the wild-type tyrosine or the mutant phenylalanine is circled. The dashed lines indicate the location of proteasomal cuts as deduced from the data shown in panels **C**–**H**. (**B**) NS3(Wt)$_{1062–1095}$ polypeptide (black bars) and NS3(Mut)$_{1062–1095}$ polypeptide (white bars) were digested for 1, 2, 4, and 8 hours with 20S immunoproteasomes. Kinetic analysis of polypeptide substrate turnover is indicated. (**C**–**H**) Kinetic analysis of cleavage product generation. Relative abundances of cleavage products derived from immunoproteasomal digestion of NS3(Wt)$_{1062–1095}$ polypeptide (filled circles) and NS3(Mut)$_{1062–1095}$ polypeptide (open circles) are plotted. The relative abundance of the NS3$_{1073–1081}$ epitope could not be assessed due to formation of disulfide bonds that interfered with mass spectrometry analysis.

a carboxyterminally elongated NS3 epitope. Finally, cleavage product NS3$_{1084–1095}$ was liberated from both the NS3(Wt)$_{1062–1095}$ and NS3(Mut)$_{1062–1095}$ polypeptide substrates in comparable amounts (Figure 2H), indicating that the proteasomal cut between amino acid 1083 and 1084 was not affected by the NS3$_{1082}$ mutation.

In summary, an incorrect carboxyterminus of the NS3$_{1073–1081}$ epitope appeared to be generated from the NS3(Mut)$_{1062–1095}$ but not from the NS3(Wt)$_{1062–1095}$ polypeptide substrate.

NS3$_{1073–1081}$-specific CD8$^+$ T cells recognize aminoterminally, but not carboxyterminally elongated forms of the minimal optimal epitope. To study recognition of the processing products by CD8$^+$ T cells, we generated NS3$_{1073–1081}$-specific CD8$^+$ T cell lines from the blood of HCV-recovered patients. These T cell lines recognized target cells pulsed with the minimal optimal NS3$_{1073–1081}$-epitope in a standard cytotoxicity assay (Figure 3). Epitope-specific T cell lines also recognized the corresponding aminoterminally elongated peptides, but not the carboxyterminally elongated mutant and wild-type peptides (Figure 3), thereby confirming that correct carboxyterminal cleavage by the proteasome was indispensable (33, 34).

Direct biochemical and immunological detection of the NS3$_{1073–1081}$ epitope in proteasomal digests. To directly assess and quantitate the in vitro generation of the NS3$_{1073–1081}$ epitope, it was necessary to prevent the formation of disulfide bonds between the cysteine residues. We therefore synthesized a serine variant of the epitope with a cysteine to serine exchange at position NS3$_{1073}$. This NS3$_{1073–1081}$ serine variant was equally well recognized by cytotoxic T cells as the original NS3$_{1073–1081}$ epitope (Figure 4A).

When the corresponding NS3(Wt)$_{1062–1095}$ and NS3(Mut)$_{1062–1095}$ polypeptides with a serine in position NS3$_{1073}$ were subjected to digestion by constitutive proteasomes, the production of the NS3$_{1073–1081}$ serine variant could be directly assessed by mass spectrometry. As demonstrated in Figure 4B, a significantly larger amount of the NS3$_{1073–1081}$ **S**VNGVCWTV epitope was generated from the NS3(Wt)$_{1062–1095}$ polypeptide than from the NS3(Mut)$_{1062–1095}$ polypeptide. For immunological analysis, the complete 8-hour proteasomal digests were then loaded onto transporter associated with antigen processing–deficient (TAP-deficient), T2-target cells and tested for recognition by NS3$_{1073–1081}$-specific, cytotoxic T cell

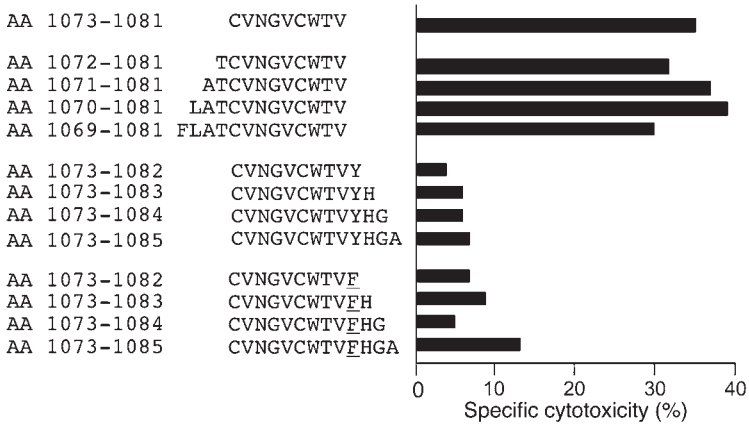

Figure 3
$NS3_{1073–1081}$ epitope–specific $CD8^+$ T cells recognize aminoterminally extended, but not carboxyterminally extended, peptides of the minimal optimal $NS3_{1073–1081}$ epitope. $NS3_{1073–1081}$–specific $CD8^+$ T cells were expanded from PBMCs of an HCV-recovered patient by several weeks of peptide stimulation and tested against amino- and carboxyterminally extended peptides in a standard ^{51}Cr-release assay. The mutant sequence at AA position 1082 is underlined.

lines. Only the wild-type and not the mutant polypeptide digests were recognized by $NS3_{1073–1081}$-specific cytotoxic T cells (Figure 4D), thus confirming the biochemical data in Figure 2.

The same qualitative results were obtained when $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ polypeptides were digested with immunoproteasome instead of constitutive proteasome. Again, a significantly larger amount of the $NS3_{1073–1081}$ **S**VNGVCWTV epitope was generated from $NS3(Wt)_{1062–1095}$ than from $NS3(Mut)_{1062–1095}$ polypeptide (Figure 4, C and E). Overall, the immunoproteasome appeared to digest the wild-type polypeptide more rapidly than the constitutive proteasome did, as indicated by a plateau-phase of epitope liberation from the wild-type polypeptide in the biochemical analysis (Figure 4C) and by the higher cytotoxicity in the immunological analysis (Figure 4E).

The HCV $NS3_{1082}$ Y/F mutant reduces the induction of HCV $NS3_{1073–1081}$-specific $CD8^+$ T cells in HLA-A2 transgenic mice. To analyze whether the altered proteasomal cleavage of the $NS3_{1082}$ Y/F mutant affected the generation of the $NS3_{1073–1081}$ epitope in vivo, we employed a humanized mouse model. Specifically, we used transgenic mice that expressed the α1 and α2 chains of the human HLA-A2 molecule and the α3 chain of the murine K^d molecule (35). Upon immunization, these mice generate T cells against the same HLA-

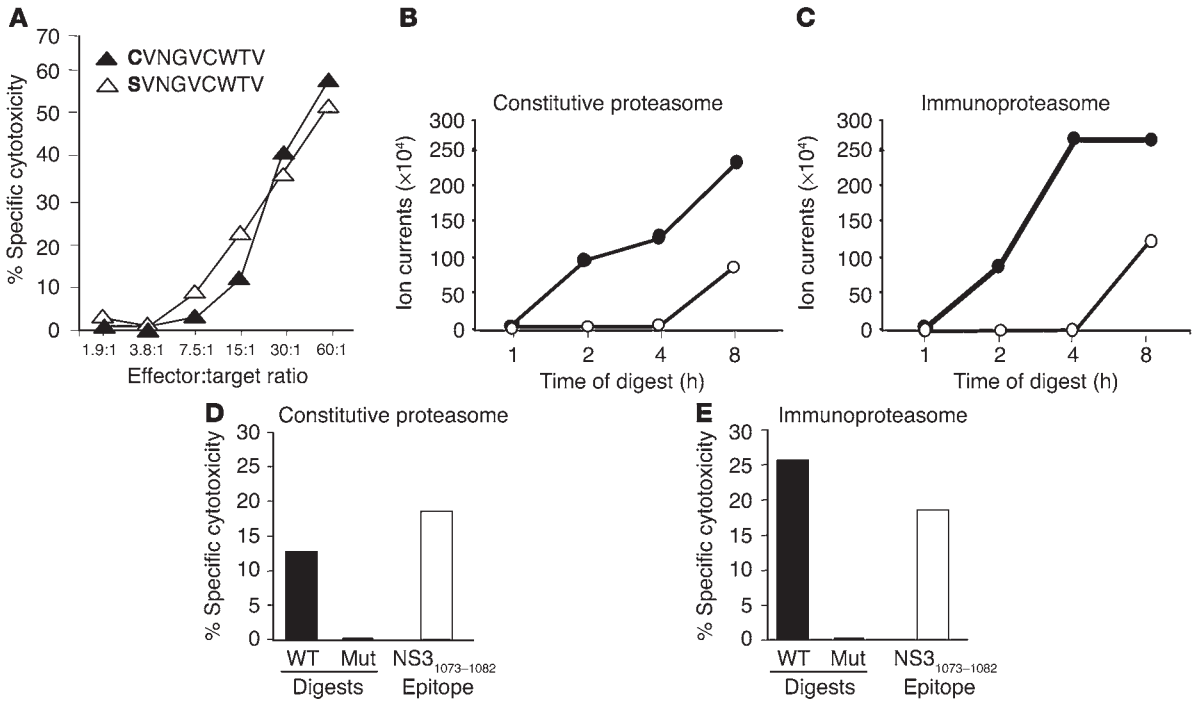

Figure 4
Biochemical and immunological analysis of proteasomal digests. (**A**) $NS3_{1073–1081}$–specific $CD8^+$ T cells lyse T2 cells loaded with either the $NS3_{1073–1081}$ serine variant **S**VNGVCWTV or the $NS3_{1073–1081}$ epitope **C**VNGVCWTV. (**B**–**C**) Generation of $NS3_{1073–1081}$ serine variant **S**VNGVCWTV from $NS3(Wt)_{1062–1095}$ (filled circles) and $NS3(Mut)_{1062–1095}$ (open circles) serine variant polypeptides $NS3_{1062–1095}$ by digestion with constitutive 20S proteasome (**B**) and by digestion with 20S immunoproteasome (**C**). (**D**–**E**) 8-hour–proteasome (**D**) and immunoproteasome (**E**) digests of $NS3(Wt)_{1062–1095}$ and $NS3(Mut)_{1062–1095}$ serine variant polypeptides were loaded onto TAP-deficient T2-target cells and incubated with $NS3_{1073–1081}$ epitope–specific, cytotoxic T cells at an effector to target ratio of 13 to 1. For comparison, 10^{-5} M $NS3_{1073–1081}$ serine variant was loaded onto T2 target cells. Only wild-type and not the mutant polypeptide digests were recognized by $NS3_{1073–1081}$ epitope–specific cytotoxic T cells.

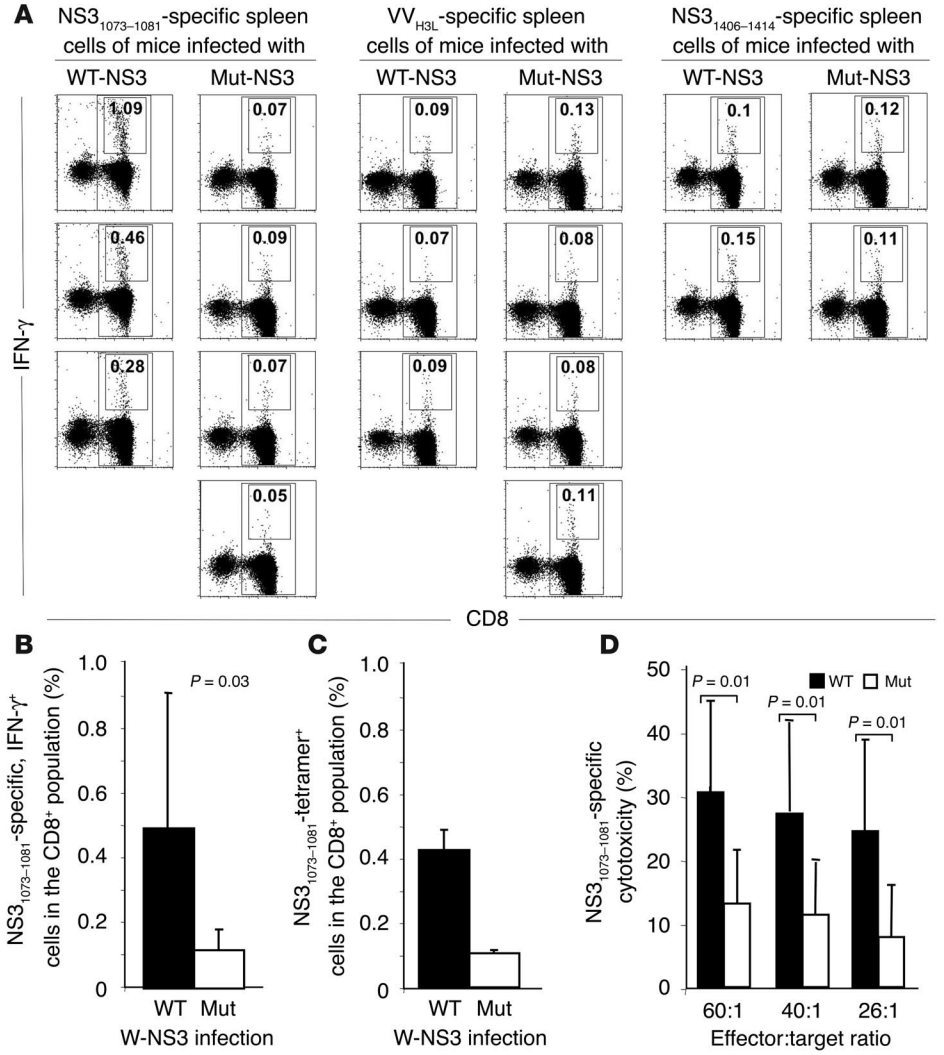

Figure 5
Immunization of HLA-A2 transgenic mice with the wild-type HCV NS3 sequence induces more IFN-γ–secreting and cytotoxic HCV $NS3_{1073–1081}$–specific CD8 T cells than immunization with the mutant HCV NS3 sequence. (**A** and **B**) Ex vivo analysis of $NS3_{1073–1081}$–specific, IFN-γ producing CD8 T cells demonstrated a significantly greater response in mice immunized with wild-type than of mice immunized with mutant NS3 encoding vaccinia virus. In contrast, the response against the vaccinia $virus_{H3L}$ (VV_{H3L}) epitope and the HCV $NS3_{1406–1414}$ epitope did not differ between both groups of mice. (**A**) Dot plots from individual mice tested in the same experiment. (**B**) Mean and standard deviation of the results of all 8 mice per group. (**C**) Frequency of $NS3_{1073–1081}$-tetramer–specific T cells is higher in mice immunized with wild-type than in mice immunized with mutant HCV NS3 sequences. Mean and standard deviation of the results of 8 mice per group are shown. (**D**) $NS3_{1073–1081}$-specific T cell lines from mice immunized with wild-type HCV NS3 sequences (WT) display greater cytotoxicity than those derived from mice immunized with mutant HCV NS3 (Mut). Mean and standard deviation of the results of 11 mice per group are shown.

A2–restricted epitopes as HLA-A2–positive humans (36). In contrast to what occurs in human studies, however, this mouse model allows the induction of T cells in the context of a single, defined HLA molecule and a single, defined viral sequence. Therefore, the HLA-A2–transgenic mouse model is not subject to the influences of additional factors such as selection pressure in the context of other HLA molecules and epitopes and/or preservation of viral replication fitness as may be operating in vivo in infected humans.

To take advantage of this model, we immunized HLA-A2–transgenic mice with vaccinia viruses that encoded full-length HCV NS3 sequences with either the Y wild type or the F mutant at amino acid position $NS3_{1082}$. Two weeks after immunization, spleen cells were isolated, and the frequency of $NS3_{1073–1081}$-specific T cells was assessed by ex vivo intracellular IFN-γ–staining and by tetramer analysis. As shown in Figure 5A for individual mice and in Figure 5, B and C, for all mice, the frequency of $NS3_{1073–1081}$-specific, IFN-γ–producing and tetramer$^+$ cells was significantly higher in mice infected with wild-type than in those infected with mutant NS3 encoding virus. In contrast, the frequency of $CD8^+$ cells that recognized either a vaccinia virus epitope or an unrelated HCV NS3 epitope did not differ between both groups of mice, thus confirming the specificity of the observation.

In separate experiments, additional immunizations with recombinant DNA-expression vectors were performed to increase the number of $NS3_{1073–1081}$-specific T cells and to establish T cell lines suitable for cytotoxicity analyses. At all effector/target ratios, cytotoxic T cell responses of mice that had been immunized with wild-type NS3 sequences were significantly stronger than cytotoxic T cell responses of mice immunized with mutant NS3 sequences (Figure 5D). Collectively, these results demonstrate that the $NS3_{1082}$ Y/F substitution reduced the generation of the $NS3_{1073–1081}$ epitope in vitro when polypeptides were digested with purified 20S proteasomes. In addition, the $NS3_{1082}$ substitution impaired the induction of epitope-specific T cells in vivo when full-length NS3 protein was endogenously expressed, ubiquitinylated, and processed by the 26S proteasome in the presence of additional cytosolic proteases.

Discussion

Most MHC class I ligands are liberated from strings of polypeptides and ubiquitinylated proteins by the proteasome, the main cytosolic protease (13). Whereas the amino-terminus of each epitope can be further defined by postproteasomal aminoexopeptidases (31, 32, 37), the carboxyterminus needs to be defined precisely with the first

cut (33, 34). Studying a single source outbreak of HCV, we identified an HCV mutation that interfered with the correct carboxyterminal cleavage of an immunodominant, HLA-A2 restricted HCV epitope from its mutated polypeptide precursor.

The emergence of viral mutations in immunogenic sequences has long been discussed as a potential immune escape mechanism. As regards HCV, sequences that do not bind to the MHC and/or the T cell receptor and thus are not recognized by HCV-specific $CD8^+$ T cells have been observed in chimpanzees and in humans (15, 16, 18). In addition, mutations that generate partial agonists or antagonists to the T cell receptor and downregulate wild-type–specific T cell responses have been described in HCV (18) as well as in hepatitis B virus (HBV) (38) and HIV infections (39). Perhaps even more efficient mechanisms of viral escape are mutations in epitope-flanking residues that interfere with antigen processing and presentation of MHC class I–restricted epitopes because, in these cases, the induction phase rather than the effector phase of HCV-specific T cell responses can be impaired. Indeed, the literature provides several examples showing that amino acid residues in the flanking regions of T cell epitopes impair proteasomal processing of those epitopes (40–43). On the other hand, there are also examples showing that extensive sequence changes in the flanking regions of other immunodominant CD8 T cell epitopes do not influence antigen processing (44). In our study, we observed impairment of antigen processing by an exchange of two very similar amino acids. Although the Y/F substitution is a conservative one, it impaired correct carboxyterminal cleavage of the $NS3_{1073–1081}$ epitope not only by constitutive proteasomes, but also by immunoproteasomes. Importantly, the same effects were observed in vitro when polypeptides were digested with purified 20S proteasomes and in vivo when full length HCV NS3 protein was endogenously expressed in an animal model, ubiquitinylated, and processed by the 26S proteasome in the presence of additional cytosolic proteases. Thus, the data demonstrate a mechanism by which a conservative HCV mutation can interfere with the induction of epitope-specific $CD8^+$ T cells.

The observation that this specific HCV mutation was also found in a substantial number of HLA-A2–negative patients is consistent with a recent report on an HIV mutation in an HLA-B51–restricted epitope, which was also less frequent in HLA-B*51-negative than in HLA-B*51-positive persons, but not completely absent in HLA-B*51-negative persons. Overall, 29% of HLA-B*51-negative persons carried the HIV mutation as compared to 98% of HLA-B*51-positive persons (43). Notably, with more than 400 patients enrolled, the HIV study was much larger than our study, and differences in the prevalence of the HIV mutation between patient subgroups were remarkable and statistically significant (43). In the HIV study as well as in our study, however, the presence of the mutation in a subpopulation of patients without the relevant HLA haplotype is consistent with the influence of multiple selection forces that drive the evolution of viral sequences in humans. These selection forces include pressure on additional, overlapping, or adjacent T cell epitopes that are presented in the context of other MHC class I and II alleles as well as selection pressure to preserve viral replication fitness (45). Because the HCV $NS3_{1073–1082}$ sequence is located directly downstream of the HCV protease domain, it is, for example, possible that the Y/F mutation affects HCV replication and was therefore also found in HLA-A2–negative patients. For these reasons, the HLA-A2–transgenic mouse model provides a valuable tool for analyzing the effect of a single mutation on the induction of epitope-specific $CD8^+$ cells in the context of a single, defined HLA molecule.

Although this study demonstrated a mechanism of altered antigen processing and impaired induction of HCV-specific T cells, significantly larger, population-based studies will be required to analyze the contribution of this mechanism to the overall selection pressure that drives HCV sequence evolution in infected patients. As a starting point for these studies, mutations in the flanking region of this particular HCV $NS3_{1073–1081}$ epitope are intriguing for several reasons. In addition to being the single most vigorously recognized $CD8^+$ T cell epitope in all published studies (4, 20–25), the $NS3_{1073–1081}$ epitope is one of only a few described epitopes that are recognized by circulating T cells as well as by nonspecifically expanded intrahepatic T cells (46). Moreover, it is also one of only two epitopes for which a TCR antagonist based on an intraepitope mutation has been described in persistently infected patients (18). The Y/F mutation in the flanking region of the epitope described here should, however, not be regarded as a main cause of HCV persistence. Rather, it should be regarded as an example of a viral escape mechanism that might also occur in the flanking regions of other $CD4^+$ and $CD8^+$ T cell epitopes. If many of these mutations occur throughout the HCV polyprotein, they may collectively contribute to the evolution of HCV quasispecies in persistently infected patients and to the characteristic weakness of the HCV-specific immune response.

Methods

Clinical samples. Sera of patients with persistent HCV infection were analyzed 18 years after an accidental single-source outbreak (genotype 1b, AJ32996) due to a contaminated anti–D immunoglobulin (20, 26). The patients gave informed consent to this analysis. Patient samples were analyzed at Medizinische Hochschule Hannover (MHH) under a protocol approved by the MHH Ethics Committee. At the time of analysis, none of the patients had developed liver cirrhosis. HCV persistence was defined by detection of serum HCV RNA by RT-PCR, and by detection of HCV antibodies by enzyme immunoassay (HCV Version 3.0; Abbott Diagnostika GmbH, Wiesbaden, Germany). HLA typing was performed with Terasaki HLA-typing trays (One Lambda Inc., Canoga Park, California, USA).

Peptides. The wild-type $NS3(Wt)_{1062–1095}$ VSTATQSFLATCVNGVCWTVYHGAGSKTLAGPKG polypeptide, the mutant $NS3(Mut)_{1062–1095}$ VSTATQSFLATCVNGVCWTV**F**HGAGSKTLAGPKG polypeptide, the $NS3_{1062–1095}$ serine variants VSTATQSFLAT**S**VNGVCWTVYHGAGSKTLAGPKG and VSTATQSFLAT**S**VNGVCWTV**F**HGAGSKTLAGPKG, the $NS3_{1073–1081}$ epitope CVNGVCWTV and the corresponding carboxyterminally and/or amino-terminally elongated peptides, the $NS3_{1073–1081}$ serine variant **S**VNGVCWTV, and the control peptides vaccinia $virus_{H3L}$ SLSAYIIRV (47) and HCV $NS3_{1406–1415}$ KLVALGINAV were synthesized using standard Fmoc methodology on an Applied Biosystems 433A automated synthesizer at >90% purity (Applied Biosystems, Darmstadt, Germany).

Detection and sequencing of HCV. Total RNA was extracted from 140 μl sera using QIAamp Viral RNA Kit (QIAGEN GmbH, Hilden, Germany), and reverse transcription was performed with Superscript II Reverse Transcriptase (Gibco BRL, Grand Island, New York, USA) and random hexamers (48, 49). HCV sequences from the highly conserved 5′ untranslated region were amplified with Taq Polymerase (Gibco BRL) in a nested PCR with prim-

ers HCV43S (CCCTGTGAGGAACT[AT]CTGTCTTCACGC) and HCV318AS (GGTGCACGGTCTACGAGACCT) and nested primers HCV78S (TCTAGCCATGGCGTTAGTCAG[CT]GA) and HCV288AS (CACTCGCAAGCACCCTATCAGGCAGT) (BioteZ Berlin-Buch GmbH, Berlin, Germany) in 1 cycle with 5 minutes at 93°C, 2 minutes at 52°C, 3 minutes at 72°C; in 35 cycles with 1 minute at 93°C, 1 minute at 52°C, 2 minutes at 72°C; and in 1 cycle with 4 minutes at 72°C. PCR products were visualized on an ethidium bromide–stained 1.5% agarose gel.

For sequencing analysis, reverse transcription of RNA isolated from the inoculum was performed with the HCV-specific primer NS3as (CAGCATGCCTCGTGACCA); reverse transcription of RNA isolated from the serum of persistently infected patients was performed with Superscript II Reverse Transcriptase (Gibco BRL) and random hexamers as previously described (48, 49). The $NS3_{987–1133}$ region containing the $NS3_{1073–1081}$ epitope was amplified with primers NS3s (GAGGCCACTATGTCCAAATG) and NS3as (CAGCATGCCTCGTGACCA) and nested primers NS3s-nested (GTAGAGCCCGTCGTCTTCTC) and NS3as-nested (GCCTCGTGACCAAGTAAA-GG). PCR conditions for the outer primer pair were 1 cycle with 2 minutes at 94°C; 30 cycles with 30 seconds at 94°C, 90 seconds at 50°C, and 2 minutes at 72°C; and 1 cycle with 4 minutes at 72°C. PCR conditions for the inner primer pair were 1 cycle with 2 minutes at 94°C; 30 cycles with 30 seconds at 94°C, 90 seconds at 59°C, and 2 minutes at 72°C; and 1 cycle with 4 minutes at 72°C. PCR products were either sequenced directly using the ABI PRISM BigDye Terminator Cycle Ready Reaction Kit and the ABI PRISM 310 sequencer (PerkinElmer, Rodgau-Jügesheim, Germany) or cloned into pCRII Vector (Topo TA Cloning Kit; Invitrogen, Carlsbad, California, USA) for sequencing of molecular clones. Sequence analysis was performed with software Factura and Sequence Navigator (PerkinElmer).

Generation of recombinant vaccinia viruses encoding wild-type and mutant HCV NS3. The HCV clone HCV-AD78P1 (Genbank number AJ132997) (50) was used as a template to amplify the full-length NS3 sequence by PCR using primers NS3FL-s (CCGCTAGCCACCATGGCGCCCATCACGGCCTATTCC; nt 3081–3101) and NS3FL-as (CCGCGGCCGCTTAGGTGACGACCTCCAGGTCAGC; nt 4974–4954) under the following PCR conditions: 1 cycle with 2 minutes at 95°C; 5 cycles with 30 seconds at 94°C, 1 minute at 52°C, and 4 minutes at 72°C; followed by 25 cycles with 30 seconds at 94°C, 1 minute at 60°C, and 4 minutes at 72°C; and 1 cycle with 4 minutes at 72°C. The primer pair included a KpnI (5′) and NotI (3′) restriction site, respectively, for cloning into the pEF1/myc-His vector (Invitrogen). Site-directed mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) and primers GCTGGAGTGTCTACCATGGCGCTGGC and GCCAGCGCCATGGTAGACAGTCCAGC to substitute the tyrosine for phenylalanine at position $NS3_{1082}$. NS3-sequences were then subcloned into plasmid p7.5K131 (51) using EcoRI restriction sites to generate the recombinant vaccinia viruses $VV_{NS3\text{-}Wt}$ and $VV_{NS3\text{-}Mut}$ by homologous recombination with the Copenhagen strain and selection on 143TK$^-$ cells. Synthesis of HCV-NS3–specific mRNA was confirmed by Northern blot analysis of CV-1 cells infected with vaccinia viruses (MOI 10) using radioactively labeled HCV NS3-cDNA and standard techniques (52). HCV-NS3 expression of wild-type and mutant NS3-encoding vaccinia virus was comparable as determined by flow cytometry (not shown).

Proteasome purification and peptide digestion. Constitutive 20S proteasomes were isolated from HepG2 human hepatoma cells and murine MEC-18 cells. Immunoproteasomes were isolated from HepG2 that had been cultured with 200 U/l human IFN-γ (Roche Diagnostics GmbH, Mannheim, Germany) for 72 hours and from MEC-18 cells that had been transfected with LMP2, LMP7, and MECL-1 under a tetracycline-regulated promoter (10). The purity of isolated proteasomes was greater than 90% (11). Twenty micrograms of wild-type and mutant $NS3_{1062–1095}$ peptides were incubated with 2 μg purified proteasomes in 150 μl assay buffer (20 mM Hepes/KOH, pH 7.8, 2 mM MgAc2, 1 mM dithiothreitol) at 37°C. The reaction was terminated by the addition of 0.1% trifluoroacetic acid (TFA). Forty microliters of the digests were separated by reversed-phase chromatography on a μRPC C2/C18 2.1/10 column (Pharmacia Biotech, Freiburg, Germany) and analyzed online with an ion trap mass spectrometer (LCQ; Electron Corp., Dreieich, Germany) with an electrospray ion source. Peptides were identified by tandem mass spectrometry (MS/MS) experiments and the amount of generated $NS3_{1073–1081}$ epitope was calculated by comparison to a defined amount of synthetic $NS3_{1073–1081}$ peptide.

In vivo induction of HCV-specific CD8$^+$ T cells in HLA-A2–transgenic mice. AAD mice, which express the α1 and α2 domains of the HLA-A2.1 molecule and the α3 domain of the murine H-2D^d molecule (35), were used to test the in vivo effect of the HCV mutation on the induction of $NS3_{1073–1081}$-specific T cells. The animal protocol was approved by the National Institute for Diabetes, Digestive and Kidney Diseases (NIDDK) Animal Care and Use Committee.

For ex vivo analysis of $NS3_{1073–1081}$-specific T cells, 6- to 8-week-old AAD mice were intraperitoneally injected with 10^7 PFU recombinant vaccinia virus ($VV_{NS3\text{-}Wt}$ and $VV_{NS3\text{-}Mut}$ respectively) in 200 μl PBS. Spleens were isolated two weeks after immunization, injected with 400 μg/ml of Liberase CI (Roche Diagnostics, Indianapolis, Indiana, USA), incubated at 37°C for 30 min and forced through a cell strainer (Falcon; BD Biosciences, Franklin Lakes, New Jersey). Single-cell suspensions were subsequently incubated with purified anti-mouse CD16/CD32 (FcgIII/IIR; BD Pharmingen, San Diego, California, USA) for 15 minutes at 4°C, then washed with PBS/2% FBS and stained with the HLA-A2/HCV $NS3_{1073}$ tetramer (NIAID Tetramer Facility, Atlanta, Georgia, USA) for 30 minutes at room temperature. After two washes, cells were stained with FITC-conjugated anti-CD8 for an additional 30 minutes at 4°C, washed again, and resuspended in 500 l PBS/0.5% paraformaldehyde (PFA) for flow cytometry.

For analysis of cytokine production, CD8$^+$ T cells were isolated from spleen cells using MACS CD8$^+$ T Cell Isolation Kit and Columns (Miltenyi Biotec, Auburn, California, USA), according to the manufacturer's instructions. C1R-AAD cells (10^6) (35) that had been pulsed overnight with 10 g/ml of either HCV $NS3_{1073–1081}$, VV $H3L_{35–43}$, or HCV $NS3_{1406–1415}$ peptide were used to stimulate 2×10^6 purified CD8$^+$ T cells for 12 hours in 1 ml RPMI1640 containing 10% fetal bovine serum (BioWhittaker, Walkersville, Maryland, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (standard medium), additionally supplemented with 50 U/ml IL-2, 10 g/ml brefeldin A (BD Pharmingen), 2 l anti-CD28, and 2 l anti-CD49d. Cells were then pelleted, washed with PBS/2% FBS, and stained at 4°C for 30 minutes with FITC-conjugated anti-CD8 (BD Pharmingen). After two washes in PBS, cells were fixed with Cytofix/Cytoperm (BD Pharmingen) at 4°C for 30 minutes. Thereafter, cells were washed twice with 1× Perm/Wash buffer (BD Pharmingen) and stained at 4°C for 30 minutes with PE-conjugated anti–IFN-γ or

PE-conjugated IgG1 isotype control. After one additional wash in 1× Perm/Wash buffer, cells were resuspended in PBS and analyzed using a Becton-Dickinson FACSCalibur with CellQuest (BD, San Jose, California, USA) and FlowJo software (Tree Star, San Carlos, California, USA). At least 10,000 events were acquired in a forward and side-scatter gate set to exclude cell debris.

For the generation of T cell lines and for in vitro analysis of cytotoxicity, mice were immunized twice with 10^7 PFU recombinant vaccinia virus ($VV_{NS3\text{-}Wt}$ or $VV_{NS3\text{-}Mut}$) in 200 μl PBS at 4-week intervals. An additional intramuscular immunization with NS3-Wt and NS3-Mut encoding pEF1/myc-His plasmids (Invitrogen) was performed prior to the vaccinia virus immunization in some experiments, but did not further enhance the $NS3_{1073–1081}$-specific T cell response. Spleen cells were harvested 7 days after the last immunization and stimulated in T-25 flasks (3×10^7 cells/flask) in standard medium containing 50 μg/ml synthetic $NS3_{1073–1081}$ peptide. On day 2 of culture, 10% Rat-T-Stim (Collaborative Biomedical Products, Bedford, Massachusetts, USA) was added. On day 7, a standard 51Cr release assay was performed using $NS3_{1073–1081}$-peptide–pulsed, ^{51}Cr-labeled CIR-AAD cells as target cells, and a 40-fold excess of unlabeled CIR-AAD as previously described (36). Percent specific lysis was calculated as (experimental release – spontaneous release) ×100/(maximum release – spontaneous release), in which spontaneous and maximum release reflect target cell lysis in the absence of effector cells and in the presence of 10% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA), respectively. Nonspecific lysis in the absence of peptide was less than 10% in all assays.

Cytotoxicity assay using human T cell lines. HCV $NS3_{1073–1081}$–specific T cell lines were used to detect the $NS3_{1073–1081}$ epitope in the proteasome digests. For this purpose, HCV $NS3_{1073–1081}$–specific, cytotoxic $CD8^+$ T cell lines were established by repetitive $NS3_{1073–1081}$ peptide stimulation (20) from PBMCs of HCV-recovered patients. HCV-recovered patients were followed in the Liver Diseases Section, NIDDK, NIH, and gave informed consent according to a protocol approved by the NIDDK Institutional Review Board. HCV-specific T cell lines were tested in a serum-free ^{51}Cr-release assay (20) against TAP-deficient T2 target cells that had either been loaded with 1 M peptide overnight or incubated with 8-hour–proteasome digests for 12 hours in serum-free RPMI 1640 medium prior to labeling with 50 Ci ^{51}Cr. Only $NS3_{1073–1081}$-specific T cell lines with a sensitivity level of at least 0.1 M peptide were tested against target cells loaded with the proteasomal digests.

Statistical analysis. Student's *t* test (two-tailed) was used to compare the frequency of $NS3_{1073–1081}$-specific cells with their CTL activity in mice immunized with recombinant vaccinia virus expressing wild-type or mutant NS3, respectively. The Fisher exact probability test (two-tailed) was used to compare the frequency of the Y/F mutation in HLA-A2–positive and –negative patients.

Acknowledgments

The authors thank L. Müller and S. Bigl (Landesuntersuchungsanstalt, Chemnitz, Germany) for patient samples; M. Roggendorf (Universitätsklinikum Essen, Germany) for PCR products of the infectious source; and A. Elstner for expert technical assistance. The HLA-A2 tetramer was produced at the National Institute of Allergy and Infectious Diseases Tetramer Facility of the NIH AIDS Research and Reference Reagent Program. This study was supported by grant Se885/1-1 of the Deutsche Forschungsgemeinschaft, Bonn, Germany, to U. Seifert; grant 1297 (01KI 9659/5 Pathogenesis of HCV infection) of the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF) to B. Rehermann; Sonderforschungsbereich 421 to P. Kloetzel and H. Hengel; and by the NIDDK, NIH, Department of Health and Human Services (DHHS) intramural research program.

Received for publication January 5, 2004, and accepted in revised form May 18, 2004.

Address correspondence to: Barbara Rehermann, Liver Diseases Section, NIDDK, NIH, DHHS, 10 Center Drive, Room 9B16, Bethesda, Maryland 20892, USA. Phone: (301) 402-7144; Fax: (301) 402-0491; E-mail: Rehermann@nih.gov.

Heiner Wedemeyer's present address is: Department of Gastroenterology, Hepatology and Endocrinology, Medizinische Hochschule Hannover (MHH), Hannover, Germany.

Thomas Ruppert's present address is: Zentrum für Molekulare Biologie Heidelberg, University of Heidelberg, Heidelberg, Germany.

Alice Sijts's present address is: David H. Smith Center for Vaccine Biology and Immunology, Aab Institute for Biomedical Sciences, University of Rochester Medical Center, Rochester, New York, USA.

1. Shoukry, N., et al. 2003. Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J. Exp. Med.* **197**:1645–1655.
2. Grakoui, A., et al. 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science.* **302**:659–662.
3. Diepolder, H.M., et al. 1995. Possible mechanism involving T lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet.* **346**:1006–1007.
4. Lechner, F., et al. 2000. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur. J. Immunol.* **30**:2479–2487.
5. Thimme, R., et al. 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J. Exp. Med.* **194**:1395–1406.
6. Pamer, E., and Cresswell, P. 1998. Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* **16**:323–358.
7. Khan, S., et al. 2001. Immunoproteasomes largely replace constitutive proteasomes during an antiviral and antibacterial immune response in the liver. *J. Immunol.* **167**:6859–6868.
8. Schwarz, K., et al. 2000. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. *J. Immunol.* **165**:768–778.
9. Groettrup, M., Khan, S., Schwarz, K., and Schmidtke, G. 2001. Interferon-gamma inducible exchanges of 20S proteasome active site subunits: why? *Biochimie.* **83**:367–372.
10. Sijts, A.J., et al. 2000. Efficient generation of a hepatitis B virus cytotoxic T lymphocyte epitope requires the structural features of immunoproteasomes. *J. Exp. Med.* **191**:503–514.
11. Groettrup, M., et al. 1995. The interferon-gamma-inducible 11 S regulator (PA28) and the LMP2/LMP7 subunits govern the peptide production by the 20 S proteasome in vitro. *J. Biol. Chem.* **270**:23808–23815.
12. Sijts, A.J., et al. 2000. MHC class I antigen processing of an adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. *J. Immunol.* **164**:4500–4506.
13. Kloetzel, P.M. 2001. Antigen processing by the proteasome. *Nat. Rev. Mol. Cell Biol.* **2**:179–187.
14. Neumann, A.U., et al. 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science.* **282**:103–107.
15. Weiner, A., et al. 1995. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc. Natl. Acad. Sci. U. S. A.* **92**:2755–2759.
16. Erickson, A.L., et al. 2001. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity.* **15**:883–895.
17. Farci, P., et al. 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science.* **288**:339–344.
18. Chang, K.M., et al. 1997. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C

virus. *J. Clin. Invest.* **100**:2376–2385.
19. Tsai, S.L., et al. 1998. Hepatitis C virus variants circumventing cytotoxic T lymphocyte activity as a mechanism of chronicity. *Gastroenterology.* **115**:954–965.
20. Takaki, A., et al. 2000. Cellular immune responses persist, humoral responses decrease two decades after recovery from a single source outbreak of hepatitis C. *Nat. Med.* **6**:578–582.
21. Lechner, F., et al. 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. *J. Exp. Med.* **191**:1499–1512.
22. Gruener, N.H., et al. 2000. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J. Infect. Dis.* **181**:1528–1536.
23. Cerny, A., et al. 1995. Cytotoxic T lymphocyte response to hepatitis C virus - derived peptides containing the HLA A2.1 binding motif. *J. Clin. Invest.* **95**:521–530.
24. Cucchiarini, M., et al. 2000. Vigorous peripheral blood cytotoxic T cell response during the acute phase of hepatitis C virus infection. *Cell Immunol.* **203**:111–123.
25. Prezzi, C., et al. 2001. Virus-specific CD8(+) T cells with type 1 or type 2 cytokine profile are related to different disease activity in chronic hepatitis C virus infection. *Eur. J. Immunol.* **31**:894–906.
26. Wiese, M., Berr, F., Lafrenz, M., Porst, H., and Oesen, U. 2000. Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in Germany: a 20-year multicenter study. *Hepatology.* **32**:91–96.
27. Wertheimer, A.M., et al. 2003. Novel CD4+ and CD8+ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. *Hepatology.* **37**:577–589.
28. Wedemeyer, H., et al. 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J. Immunol.* **169**:3447–3458.
29. Seifert, U., et al. 2003. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat. Immunol.* **4**:375–379.
30. Kuckelkorn, U., et al. 2002. Link between organ-specific antigen processing by 20S proteasomes and CD8(+) T cell-mediated autoimmunity. *J. Exp. Med.* **195**:983–990.
31. Mo, X.Y., Cascio, P., Lemerise, K., Goldberg, A.L., and Rock, K. 1999. Distinct proteolytic processes generate the C and N termini of MHC class I-binding peptides. *J. Immunol.* **163**:5851–5859.
32. Beninga, J., Rock, K.L., and Goldberg, A.L. 1998. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem.* **273**:18734–18742.
33. Niedermann, G., et al. 1995. Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. *Immunity.* **2**:289–299.
34. Craiu, A., Akopian, T., Goldberg, A., and Rock, K.L. 1997. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proc. Natl. Acad. Sci. U. S. A.* **94**:10850–10855.
35. Newberg, M.H., et al. 1996. Importance of MHC class 1 alpha2 and alpha3 domains in the recognition of self and non-self MHC molecules. *J. Immunol.* **156**:2473–2480.
36. Wedemeyer, H., et al. 2001. Oral immunization with HCV-NS3-transformed salmonella: induction of HCV-specific CTL in a transgenic mouse model. *Gastroenterology.* **121**:1158–1166.
37. Stoltze, L., et al. 2000. Two new proteases in the MHC class I processing pathway. *Nat. Immunol.* **1**:413–418.
38. Bertoletti, A., et al. 1994. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature.* **369**:407–410.
39. Klenerman, P., et al. 1994. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature.* **369**:403–407.
40. Del Val, M., Schlicht, H.J., Ruppert, T., Reddehase, M.J., and Koszinowski, U.H. 1991. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell.* **66**:1145–1153.
41. Theobald, M., et al. 1998. The sequence alteration associated with a mutational hotspot in p53 protects cells from lysis by cytotoxic T lymphocytes specific for a flanking peptide epitope. *J. Exp. Med.* **188**:1017–1028.
42. Beekman, N.J., et al. 2000. Abrogation of CTL epitope processing by single amino acid substitution flanking the C-terminal proteasome cleavage site. *J. Immunol.* **164**:1898–1905.
43. Moore, C.B., et al. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science.* **296**:1439–1443.
44. Brander, C., et al. 1999. Efficient processing of the immunodominant, HLA-A*0201-restricted human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitope despite multiple variations in the epitope flanking sequences. *J. Virol.* **73**:10191–10198.
45. Friedrich, T.C., et al. 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. *Nat. Med.* **10**:275–281.
46. Koziel, M.J., et al. 1995. HLA class I-restricted cytotoxic T lymphocytes specific for hepatitis C virus. Identification of multiple epitopes and characterization of patterns of cytokine release. *J. Clin. Invest.* **96**:2311–2321.
47. Drexler, I., et al. 2003. Identification of vaccinia virus epitope-specific HLA-A*0201-restricted T cells and comparative analysis of smallpox vaccines. *Proc. Natl. Acad. Sci. U. S. A.* **100**:217–222.
48. Rehermann, B., et al. 1996. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response, disease activity and viral load in patients with chronic hepatitis C virus infection. *J. Clin. Invest.* **98**:1432–1440.
49. Rehermann, B., et al. 1996. Differential cytotoxic T lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. *J. Virol.* **70**:7092–7102.
50. Rispeter, K., Lu, M., Lechner, S., Zibert, A., and Roggendorf, M. 1997. Cloning and characterization of a complete open reading frame of the hepatitis C virus genome in only two cDNA fragments. *J. Gen. Virol.* **78**:2751–2759.
51. Schlicht, H.J., and Schaller, H. 1989. The secretory core protein of human hepatitis B virus is expressed on the cell surface. *J. Virol.* **63**:5399–5404.
52. Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press. Plainview, New York, USA. 37–84.