The Sequence Alteration Associated with a Mutational Hotspot in p53 Protects Cells From Lysis by Cytotoxic T Lymphocytes Specific for a Flanking Peptide Epitope

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Summary

A high proportion of tumors arise due to mutation of the p53 tumor suppressor protein. A p53 hotspot mutation at amino acid position 273 from R to H, flanking a peptide epitope that spans residues 264-272, renders cells resistant to killing by human histocompatibility leukocyte antigen (HLA)-A*0201-restricted cytotoxic T lymphocytes (CTLs) specific for this epitope. Acquisition of the R to H mutation at residue 273 of the human p53 protein promotes tumor growth in vivo by selective escape from recognition by p53.264–272 peptide-specific CTLs. Synthetic 27-mer p53 polypeptides covering the antigenic nonamer region 264–272 of p53 were used as proteasome substrates to investigate whether the R to H mutation at the P1’ position of the COOH terminus of the epitope affects proteasome-mediated processing of the protein. Analysis of the generated products by tandem mass spectrometry and the kinetics of polypeptide processing in conjunction with CTL assays demonstrate that the R to H mutation alters proteasomal processing of the p53 protein by inhibiting proteolytic cleavage between residues 272 and 273. This prevents the release of the natural CTL epitope that spans flanking residues 264–272 as well as a putative precursor peptide. These results demonstrate that mutation of p53 not only leads to malignant transformation but may also, in some instances, affect immune surveillance and should be considered in the design of cancer vaccines.

Key words: p53 • tumor antigens • cytotoxic T lymphocytes • antigen processing • proteasomes
Peptides that are presented on the cell surface by class I MHC molecules for recognition by CTLs most often are derived from proteolytic processing of cellular proteins by the multicatalytic proteasome complex (14–16). Proteolytic degradation of p53 has been found to be dependent on proteasomal processing (17–20). Proteasome-generated peptide products are subsequently translocated into the endoplasmic reticulum (ER) by the transporters associated with antigen processing, where they are loaded into the peptide-binding groove of nascent class I molecules (21–23). In this report, we demonstrate that proteasomal processing of the natural A*0201-restricted CTL epitope p53.264–272 and of a putative precursor peptide is profoundly affected by a mutational p53 hotspot (R to H) at the COOH-terminal flanking residue 273. As a consequence, target cells that overexpress p53 harboring the 273 R to H mutation are not susceptible to in vitro and in vivo lysis by A*0201-restricted CTLs specific for the flanking p53 epitope, 264–272. To our knowledge, these experiments demonstrate the first example of a naturally occurring mutation flanking a CTL epitope and affecting the ability of the proteasome to generate a defined MHC class I ligand.

Materials and Methods

Mice. The derivation of homozygous A2/Kb-Tg mice expressing a chimeric transgene that consists of the α1 and α2 domains of A*0201 and the α3 domain of H-2Kb has been described previously (4, 8). Mice were propagated and maintained under specific pathogen-free conditions. All experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Peptides. 27-mer polypeptides spanning residues 256–282 of Hu WT (TLEDSSGNLLGRNSFEVRVCAACPRGDR) and mutant (273 R to H) p53 were synthesized on an automated peptide synthesizer (432A; PE Applied Biosystems, Foster City, CA). Peptides p53.264–272 (LLGRNSFEVR) and 260–272 (SSGNNLLGRNSFEV) were synthesized by the core facility of TSRI (430A synthesizer; PE Applied Biosystems). Purity of synthetic peptides was ascertained by reverse phase (RP)-HPLC and mass spectrometry (MS). Amino acid residues are given in single letter code.

Cell Lines. Previously described cell lines and transfectants used in these studies included T1 and T2 cells (24), the naturally A*0201-expressing, p53-deficient osteosarcoma line Saos-2, and the same cells transfected with Hu p53 genes harboring mutations at residue 143 (V to A), 175 (R to H), and 273 (R to H) (4, 25). M urine thymoma lines (EL4) transfected with A*0201/Kb (EA2K*) or both A*0201/Kb and a Hu p53 gene harboring an R to H mutation at residue 273 (EA2K*.1p53) have been previously described (4). CTL lines were used as effector cells at the indicated E/T ratios in 51Cr-release assays (4).

Viral p53 R recombinants. The derivation of parental canarypox and canarypox virus p53 recombinants expressing Hu p53 without (WT) or with mutations at residue 175 (R to H) or 273 (R to H) has been previously described (5). Saos-2 targets were labeled with 51Cr and infected with parental and recombinant canarypox viruses at an infectious dose of 20 PFU/cell. Cells were washed after 1 h and incubated for another 3-h period before responder CTLs were added for a 3-h 51Cr-release assay (4). Recombinant vaccinia viruses (rVV) which contain minigenes encoding either p53.149–157 (rVV-ES149) or p53.264–272 (rVV-ES264) downstream of the ER insertion sequence of E19 (26), and rVV-VPE16 that expresses the gp160 gene of HIV type 1 (27), were provided by Drs. Jack Bennink and Jonathan Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Protection of Tumor Growth in A2/Kb-Tg Mice. A2/Kb-Tg mice were either nonimmunized or immunized intravenously with 5 × 106 PFU of rVV-ES149, rVV-ES264, or rVV-VPE16. 2 wk later, mice were challenged subcutaneously in the right flank with 5 × 105 EA2K* or EA2K*.1p53 tumor cells. The size of tumors was measured by the formula (a × b/2), where a is the horizontal and b the vertical diameter of the tumor mass as determined by calipers. Three mice were used for each experimental and control group.

Purification of 20S Proteasomes. 20S proteasomes were purified from Hu T1 cells by standard procedures (28–31). Cell pellets were lysed in lysate buffer (80 mM KAc, 5 mM MgAc2, 10 mM Hepes, pH 7.2, and 0.1% Triton X-100), dounce homogenized, spun at 40,000 g for 20 min. Supernatant was adsorbed to equilibrated DEAE-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) for 45 min. Bound protein was removed with wash buffer (80 mM KAc, 5 mM MgAc2, and 10 mM Hepes, pH 7.2), and bound protein was eluted (500 mM KAc, 5 mM MgAc2, and 10 mM Hepes, pH 7.2). The protein containing eluate was concentrated by ultrafiltration, loaded on a 10–40% sucrose gradient in wash buffer, and ultracentrifuged (40,000 rounds/min in an SW40 Ti rotor for 15.5 h; Beckman, Fullerton, CA). Gradient fractions containing 20S proteasome complexes were identified by enzymatic assays using fluorogenic substrate peptides, pooled, and concentrated for chromatography on a MonoQ HR 5/5 anion exchange column (Amersham Pharmacia Biotech; eluent A: 1 M KCl, 5 mM MgCl2, and 10 mM Hepes, pH 7.2; eluent B: 1 M KCl, 5 mM MgCl2, and 10 mM Hepes, pH 7.2; linear gradient). The 20S proteasomes obtained were again identified by enzymatic activity and eluted as single peak upon rechromatography. Purity of 20S proteasome preparations was 90% as assessed by Coomassie-stained PAGE gels. Two-dimensional gel electrophoresis revealed that all detectable proteasomal subunits were present in 20S proteasomes purified from the Hu T1 cell line.

In Vitro Digestion of Polypeptide Substrates by Purified 20S Proteasomes and Recognition by CTLs. 20S proteasomes were incubated with peptides synthesized on an automated peptide synthesizer (432A; PE Applied Biosystems, Foster City, CA). Gradient fractions containing 20S proteasome complexes were identified by enzymatic assays using fluorogenic substrate peptides, pooled, and concentrated for chromatography on a MonoQ HR 5/5 anion exchange column (Amersham Pharmacia Biotech; eluent A: 100 mM KCl, 5 mM MgCl2, and 10 mM Hepes, pH 7.2; eluent B: 1 M KCl, 5 mM MgCl2, and 10 mM Hepes, pH 7.2; linear gradient). The 20S proteasomes obtained were again identified by enzymatic activity and eluted as single peak upon rechromatography. Purity of 20S proteasome preparations was 90% as assessed by Coomassie-stained PAGE gels. Two-dimensional gel electrophoresis revealed that all detectable proteasomal subunits were present in 20S proteasomes purified from the Hu T1 cell line.
Pharmacia Biotech) and eluted with a gradient of 15–65% of eluent B (70% acetonitrile in 0.05% TFA) in eluent A (0.05% TFA) in 33 min at a flow rate of 50 μl/min (29–31). Mass analysis of peptides was performed online by a tandem quadrupole mass spectrometer (TSQ 7000; Finnigan MAT, San Jose, CA) equipped with an electrospray ion source. Each scan was acquired over the range mass/charge (m/z) 300–1150 every 2 s (29–31). Peptides were identified by their molecular mass calculated according to the mass/charge (m/z) of single or multiple charged ions. Abundant peptides with signal intensities of at least threefold above background were sequenced by tandem MS (MS/MS) after fragmentation of the relevant peptides with argon atoms (30). Peptide sequence was determined by coelution of synthetic peptides and sequenced by MS/MS (30). Ions of m/z corresponding to the relevant double protonated peptides were fragmented by argon atoms. Collision-activated dissociation fragments of relevant m/z and derived from the pooled antigenic HPLC fractions were compared with those obtained after argon atom-mediated fragmentation of the corresponding synthetic peptides.

HPLC Separation of Processed Peptides, Identification by CTLs, and Sequence Analysis of Antigenic Peptides by MS/MS. 50 μl of the bulk 205 proteasome-degraded peptide products were separated by RPHPLC into 1- (50 μl) and 0.5- (25 μl) min fractions (30). 32P-labeled T2 target cells were assayed for 40 min with half of each of the HPLC fractions in serum-free RPMI 1640 medium supplemented with 5% vol/vol BSA and Hu ϕ2b at 10 μg/ml. CTL A2 264 were used as effector cells at an E/T ratio of 20:1 in a 6-h 32P-release assay. The antigenic HPLC fractions were dried and resuspended in 50% methanol/1% acetic acid. Antigenic peptides present in the pooled HPLC fractions were identified by coelution of synthetic peptides and sequenced by MS/MS (30). Ions of m/z corresponding to the relevant double protonated peptides were fragmented by argon atoms. Collision-activated dissociation fragments of relevant m/z and derived from the pooled antigenic HPLC fractions were compared with those obtained after argon atom-mediated fragmentation of the corresponding synthetic peptides.

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HPLC Separation of Natural Peptide Extracts and Reconstitution of CTL Lysis. 1 ml of resuspended natural peptide extract or 100 pmol of either the p53.264–272 or the p53.260–272 synthetic peptides was separated by RP-HPLC at a flow rate of 50 μl/min and eluted with a gradient of 20% of eluent B (70% acetonitrile in 0.05% TFA) in eluent A (0.05% TFA) in 32 min, a gradient of 20–48% of eluent B in eluent A in 74 min, and a gradient of 48–95% of eluent B in eluent A in 4 min. HPLC fractions were collected from 32 to 76 min (fractions 1–11; 4 min fractions at 200 μl/ fraction) and from 76 min onwards (fractions 12–44; 1 min fractions at 50 μl/fraction). 32P-labeled T2 target cells were pulsed for 40 min in serum-free RPMI 1640 medium supplemented with 5% vol/vol BSA and Hu ϕ2b at 10 μg/ml with 100 μl (fractions 4–11) or 25 μl (fractions 12–44) of fractions derived from the HPLC separation of the natural peptide extract. The corresponding volumes of fractions derived from the HPLC separations of synthetic p53.264–272 and p53.260–272 peptides and used to pulse T2 targets were 8 (fractions 4–11) and 2 μl (fractions 12–44). CTL A2 264 were used as effector cells at an E/T of 20:1 in a 5.5-h 32P-release assay. Antigenic fractions obtained from the HPLC-separated natural peptide extract were pooled and half of the pooled natural peptides was used for rechromatography by RP-HPLC. HPLC conditions remained almost the same, yet 0.5-min fractions were collected from 56 min onwards (fractions 10–44 at 25 μl/fraction). The amount of synthetic p53.264–272 and p53.260–272 peptides used for the second HPLC separations was 60 pmol. 32P-labeled T2 target cells were again pulsed for 40 min under serum-free conditions with 18 μl of fractions 10–32 derived from the rechromatography of the pooled antigenic natural HPLC fractions. The corresponding volume of fractions derived from the HPLC separations of the synthetic p53.264–272 and p53.260–272 peptides and used to pulse T2 targets were 2 μl (fractions 8, 19, 20, 29) and 0.02 μl, 0.2 μl, and 2 μl (fractions 9–18 and 21–28), respectively. CTL A2 264 were used as effector cells at an E/T of 13:1 in a 6-h 32P-release assay.

Results and Discussion

Target Cells Expressing a p53 M utational H otspot on R esidue 273 A re Not R ecognized by CTLs Specific for the Flanking Epi tope 264–272. The p53-deficient Saos-2 tumor cell line transfected with a Hu p53 gene expressing the R to H hot spot mutation at amino acid residue 273 was not susceptible to lysis by CTL A2 264 specific for the flanking peptide 264–272 (Fig. 1 A). This contrasted with the successful recognition and lysis of cells transfected with Hu p53 genes expressing mutations at amino acid positions 143 or 175 (Fig. 1 A). All three p53 transfectants were killed by CTLs specific for CTL A2 149, indicating that other epitopes of the protein were being processed and presented by the A*0201 molecule (Fig. 1 B). Furthermore, as demonstrated in Fig. 1, C and D, Saos-2 cells infected with canarypox virus recombinants expressing Hu p53 with or without mutation at residue 175 were recognized by either CTL, whereas cells infected with a canarypox virus p53 recombinant expressing the 273 R to H mutation were lysed only by CTL A2 149 and not by CTL A2 264. Taken together, these results suggested that the R to H mutation at position 273 of the p53 protein may have prevented formation of the 264–272 peptide epitope.
express the R to H mutation at residue 273 of Hu p53. Preimmunization with rVV-ES149 specifically prevented growth of EA2Kb.1p53 tumors, whereas vaccination with rVV-ES264 did not (Fig. 2). Vaccination with either virus led to equivalent priming of CTLs specific for the respective peptide (data not shown). Tumors that eventually grew out in rVV-ES149 preimmunized mice were found to have lost expression of Hu p53 protein (35). EA2Kb.1p53-derived tumors in untreated or rVV-VPE16-vaccinated A2/Kb-Tg mice progressed with the same growth rate as in rVV-ES264-immunized mice (35). Tumor growth of the parental EA2Kb cells, which expresses only low levels of murine WT p53 (8), was not prevented by either vaccination (35). These results indicate that tumors harboring the R to H mutation at residue 273 of the Hu p53 protein are able to selectively escape recognition in vivo by CTLs specific for the flanking peptide 264–272.

Figure 1. Cells expressing Hu p53 that contains an R to H mutation at residue 273 are resistant to lysis by A*0201-restricted CTLs specific for the flanking peptide epitope p53.264–272. A*0201-restricted CTL lines specific for peptide epitopes p53.264–272 (A2 264) and p53.149–157 (A2 149) (B and D) were tested for cytotoxicity at the indicated E/T ratios in 5-h (A and B) and 3-h (panel C and D) 51Cr-release assays. Targets in A and B were Saos 2 cells (○), and the same cell transfected with Hu p53 genes harboring mutations at residue 143 (V to A) (●), 175 (R to H) (△), and 273 (R to H) (▲). Targets in C and D were Saos 2 cells (○), the same cells infected with canarypox virus recombinants expressing Hu p53 without (●) or with mutations at residue 175 (R to H) (△) and 273 (R to H) (▲), and Saos 2 cells infected with the parental canarypox virus (□).

Peptides derived from 20S Proteasome Degradation of the Synthetic 27-mer Polypeptide p53.256–282 Carrying an R to H Mutation at Residue 273 Are Not Recognized by CTL A2 264. As demonstrated previously, degradation of p53 is dependent on proteasomal processing (17–20). Accordingly, one mechanism that could interfere with the availability of the 264–272 epitope would be the inability of the proteasome to produce this peptide from the 273 R to H mutant p53. This could arise if the COOH-terminal cleavage site of the 264–272 epitope was abrogated by the change from R to H at the epitope flanking residue 273. To test this hypothesis, synthetic 27-mer p53 peptides spanning residues 256–282 and containing the 264–272 peptide flanked by either the WT or mutant residue at position 273 were used as substrates for in vitro digestion by purified 20S proteasomes. T2 targets cells that express predominantly empty A*0201 molecules (24) were pulsed with the peptide digests and tested for recognition by CTL A2 264. Substantial lysis of T2 targets was observed when cells were pulsed with peptides derived from the 20S proteasome–degraded WT 27-mer, but not with those derived from degraded mutant 27-mer (Fig. 3). Recognition of the synthetic 9-mer p53.264–272 epitope as compared with the WT p53.256–282 peptide digest was 2.4 logs more efficient (2.3–2.7 logs as compared with three independent WT 27-mer peptide digests), sug-

Figure 2. Immunization of A2/Kb-Tg mice with the p53.264–272 epitope does not prevent growth of tumor cells expressing both A’0201/Kb and Hu p53 containing an R to H mutation at residue 273. A2/Kb-Tg mice were immunized intravenously with 5 × 10⁶ PFU of rVV-ES149 (○) or rVV-ES264 (●). 2 wk later (day 0), mice were challenged subcutaneously in the right flank with 5 × 10⁶ EA2Kb.1p53 (273 R to H) tumor cells. Naïvely treated or rVV-VPE16-vaccinated (encoding the HIV type 1 envelope protein gp160) A2/Kb-Tg mice behaved the same as rVV-ES264-immunized animals; tumors appeared on day 10 after challenge and progressed with the same growth rate. These negative controls are not shown for clarity.

Figure 3. Synthetic 27-mer peptide substrates containing the nonamer p53.264–272 peptide epitope. Synthetic 27-mer polypeptide substrates spanning residues 256–282 of WT (TLEDSSGNLGRNSFEV)- and 273 (R to H) p53 were incubated for 24 h with or without purified 20S proteasome. 125I-labeled T2 cells were pulsed under serum-free conditions with the indicated concentrations of WT or mutant 27-mer products that had been incubated with (●) or without (□) 20S proteasome, mutant 27-mer products that had been incubated with (▲) or without (△) 20S proteasome, the synthetic p53.264–272 peptide epitope (■), or no peptide (○). A*0201-restricted CTLs specific for the p53.264–272 peptide epitope were used as effector cells at an E/T ratio of 20:1 in a 6-h 51Cr-release assay. Peptide concentration is given as the equivalent of the input concentration of 27-mer polypeptide substrates before proteasomal degradation (nM eq). Peptide-pulsed T2 targets were not lysed by CTLs specific for the unrelated p53.149–157 peptide (data not shown).
suggesting that the 264–272 epitope did not represent an abundant product generated from the WT 27-mer by the 20S proteasome. The selective recognition of WT as opposed to mutant 27-mer peptide digests by CTL A2 264 was dependent on degradation by the 20S proteasome as no lysis was detectable when WT and mutant 27-mer peptides were incubated in the absence of the 20S proteasome.

Kinetic studies revealed that WT degradation products recognized by T cells became detectable after 1 and 2 h of incubation of the polypeptide with purified 20S proteasome (Fig. 4, A and B), reached a peak of activity after a period of between 5 and 8 h of proteasomal cleavage (Fig. 4, C and D), and could still be observed after 27 h of proteasome incubation (data not shown). These results indicate that the R to H mutational change at residue 273 affected the ability of the proteasome to release an antigenic peptide from the 27-mer that could be recognized by CTL A2 264.

The p53 Mutation at Residue 273 Abrogates the Proteasomal Cleavage Site between p53 Residues 272 and 273. To prove that the COOH-terminal cleavage site of the 264–272 epitope has indeed been abrogated by the change from R to H at flanking residue 273, the 20S proteasome-degraded (24-h) WT and mutant p53.256–282 peptide products were separated by RP-HPLC, abundant masses were identified online by MS, and the sequence of abundant peptides was confirmed by MS/MS. The majority of the dominant cleavage products generated by the 20S proteasome from the WT and mutant 27-mer polypeptides were comparable in sequence and quantity (Fig. 5, A and B). However, within the range of signal intensities of at least threefold above background, only two peptides, 260–272 and 256–272, both of which contained the COOH-terminal residue 272 of the CTL epitope 264–272, were observed (Fig. 5 A). Notably, the relative signal intensities of peptides 260–272 and 256–272 were found to be 14- and 20-fold higher, respectively, within the WT as opposed to the mutant peptide digests (Fig. 5, C and D, panels 2 and 3). The differential detectability of these peptides within the WT as opposed to the mutant 27-mer peptide digests was not due to differences in the efficiency of proteasomal degradation of either polypeptide. In general, the efficiency of 20S proteasome-induced degradation of either 27-mer peptide was >95% (Fig. 5, C and D, panel 4). Also, comparable amounts (relative signal intensity: twofold more in mutant versus WT) of the peptide 266–275, which did not use the relevant cleavage site between residues 272 and 273, were generated from the WT and mutant 27-mer (Fig. 5, C and D, panel 1).

These results indicate that the p53 R to H mutation at residue 273 retards proteolytic cleavage by the 20S proteasome between residues 272 and 273. However, we could not detect the peptide representing the CTL epitope 264–272. The proteolytic generation of the 264–272 epitope from the WT 27-mer at a quantity below the threshold of detectability by M S could have been responsible for such lack of detection, even though the bulk WT 27-mer degradation products had been recognized by CTL A2 264. The observation that the 266–275 peptide was an abundant proteasomal cleavage product of both the WT and mutant 27-mer substrate would support this interpretation, as the release of this peptide would interfere with the generation of the 264–272 CTL epitope (Fig. 5). However, it could also be possible that the peptide 260–272, which used the COOH-terminal cleavage site between residues 272 and 273, represents a precursor peptide that is subsequently trimmed in the cytosol or the ER to the size of the optimal antigenic CTL epitope 264–272. Postproteasomal trimming of the NH₂ terminus of precursor peptides has been observed previously (31, 37–40).

The p53.264–272 CTL Epitope Is Generated by Proteasomal Degradation of the WT as Opposed to the Mutant 27-mer Polypeptide Substrate. To improve the sensitivity of detection of the 264–272 peptide, the proteasome-degraded peptide products (24-h) were fractionated by RP-HPLC. T2 targets were sensitized with the individual HPLC fractions and tested for recognition by CTL A2 264. Only T2 cells that had been pulsed with fractions 24, 25, and 26 of the WT 27-mer peptide digests were recognized by CTL A2 264 (Fig. 6, A and B). The retention time of fraction 24 was matched by that of the synthetic 264–272 peptide (data not shown). WT fractions 24 and 25 were pooled and tested for the presence of the 264–272 peptide by MS/MS. Identical collision-activated dissociation fragments of ions corresponding to the double protonated 264–272 peptide were detected in the pooled WT fractions 24 and 25, and the synthetic 264–272 peptide (Fig. 6, C and D). The relevant collision-activated dissociation fragments were absent in the incubation of 20S proteasome in the absence of any polypeptide substrate served as another negative control (●). CTL A2 264 were used as effector cells at an E:T ratio of 20:1 in a 6-h 51Cr-release assay. Peptide concentration is given as the equivalent of the input concentration of 27-mer polypeptide substrates before proteasomal degradation (nM eq).

Figure 4. Kinetics of 20S proteasomal degradation of WT and mutant 27-mer p53 polypeptide substrates. WT and mutant p53.256–282 peptide substrates were incubated for 1 (A), 2 (B), 5 (C), and 8 (D) h with and without purified 20S proteasome. 32P-labeled T2 cells were pulsed under serum-free conditions with the indicated concentrations of WT 27-mer products that had been incubated with (●) and without (○) 20S proteasome. Material derived from
in pooled WT fractions 22 and 23, and in pooled mutant fractions 22 and 23 and 24 and 25 (data not shown). These results indicate that the p53.264–272 CTL epitope had been generated directly from the WT 27-mer by the 20S proteasome. Its low abundance is not unique, but a property shared with immunodominant nonamer CTL epitopes detected in 20S proteasome in vitro digests (30, 41–45). CTL assays of p53-deficient Saos-2 cells infected with rVV expressing minigenes encoding either the 149–157 or the 264–272 peptide without an ER insertion sequence revealed that the 264–272 peptide is efficiently translocated into the ER (Theobald, M., and L.A. Sherman, unpublished observation). These findings demonstrate that the p53 hotspot mutation from R to H at residue 273 prevents the 20S proteasome-mediated COOH-terminal cleavage of the flanking peptide 264–272 and its subsequent presentation by A*0201.

The more abundant flanking peptide 256–272 eluted in WT fraction 26, which was recognized poorly by CTL A2 264 (Figs. 5 A and 6 A), making it unlikely to be an independent CTL epitope. However, it is of interest that due to the HPLC conditions used, the other abundant flanking peptide 260–272 (Fig. 5 A) was identified by MS/MS to be present in the pooled WT fractions 24 and 25 (data not shown). However, the A*0201-binding affinity of the longer peptide 266–272 (GRNSFEV-R/H-VC) represents a dominant product that interferes with the formation of the 264-272 CTL epitope (panel 1). The WT p53 peptides 260-272 (SSGN LLGR N SFEV) and 256-272 (TELDSSGN LLGR N SFEV) use the COOH-terminal cleavage site of the minimal CTL epitope 264-272 between WT residues 272 and 273 (panels 2 and 3). Panel 4 shows the uncleaved WT and mutant 27-mer substrate peptides left after proteasomal degradation and used for adjusting the scale (percentage of relative intensity). Panel 5 gives the total ion current of the bulk proteasomal degradation products.

Figure 5. The p53 hotspot mutation at residue 273 (R to H) abrogates the proteasomal cleavage site between p53 residues 272 and 273. Bulk proteasome products derived after 24 h from 20S proteasome-mediated degradation of the synthetic WT (A and C) and mutant (273 R to H) (B and D) 27-mer polypeptides p53.256-282 covering the A*0201-restricted CTL epitope p53.264-272 (LLGR N SFEV) were separated by R-P-HPLC and analyzed online by MS. Abundant peptide products were sequenced by MS/MS. Cleavage products with signal intensities of at least thricefold above background and identified by mass (MS) and sequence (MS/MS) are shown in descending order according to their signal intensities (A and B, black bars). The amino acid sequence of WT (A) and mutant (B) peptide substrates with abundant (large arrows) and nonabundant (small arrows) cleavage sites is also presented. The small broken arrow (A) represents the theoretical NH2-terminal cleavage site of the nonamer CTL epitope 264-272. The quantitative comparison of some of the relevant WT (C) and mutant (D) cleavage products is shown by their elution profiles and relative signal intensities as measured by the ion current of double protonated peptide ions. As demonstrated in C and D, peptide 266-275 (GRNSFEV-R/H-VC) represents a dominant product that interferes with the formation of the 264-272 CTL epitope (panel 1). The WT p53 peptides 260–272 (SSGN LLGR N SFEV) and 256–272 (TELDSSGN LLGR N SFEV) use the COOH-terminal cleavage site of the minimal CTL epitope 264–272 between WT residues 272 and 273 (panels 2 and 3). Panel 4 shows the uncleaved WT and mutant 27-mer substrate peptides left after proteasomal degradation and used for adjusting the scale (percentage of relative intensity). Panel 5 gives the total ion current of the bulk proteasomal degradation products.
identify the natural peptide epitope presented by A*0201 and recognized by CTL A2 264, peptides were extracted from class I MHC molecules of Saos-2/143 p53 transfectants. As the 9-mer (264–272) and 13-mer (260–272) p53 peptides had an almost identical retention time under the HPLC conditions used for purifying the 20S proteasomal peptide digests, a shallower elution gradient was applied for the RP-HPLC fractionation of natural peptide extracts as well as synthetic 9-mer and 13-mer peptides. The pool of natural peptides extracted from Saos-2/143 cells was chromatographed by HPLC and 4-min fractions collected and assayed. To obtain final resolution, antigenic fractions 7 and 8 were pooled and half of the pool was rechromatographed. At the relevant retention time, 0.5 min fractions were collected in order to discriminate between the 264–272 and the 260–272 peptides. T2 cells sensitized with individual HPLC fractions served as targets for CTL A2 264.

As shown in Fig. 7 A, the peak of CTL activity obtained after rechromatography of fractions 7/8 and derived from the natural peptide extract was detected in fraction 12. This HPLC fraction had the same conductivity and retention time as compared with the antigenic fractions (12 and 13) derived from the HPLC separation of the synthetic 264–272 (LLGRNSFEV) peptide epitope. Ions of m/z = 517.2 corresponding to the double protonated 264–272 9-mer peptide were fragmented by argon atoms. Collision-activated dissociation fragments of m/z 517.2 and derived from the pooled WT fractions 24 and 25 (C) were compared with those obtained after argon atom-mediated fragmentation of the synthetic 264–272 peptide (D). In particular, fragments b8, b7, and b6, lacking the COOH-terminal residues V, E, and F, respectively, were detectable in both the pooled WT fractions 24 and 25 (C) and the synthetic 264–272 9-mer peptide (D).
cate that the Hu WT p53.264–272 as opposed to the longer 260–272 peptide is the natural epitope presented by A*0201 for recognition by CTL A2 264. Natural peptides were extracted from class I molecules of Saos-2/143 p53 transfectants. Synthetic p53.264–272 and 260–272 peptides and the natural peptide extract were fractionated by R-P-HPLC. Individual HPLC fractions were used to sensitize T2 targets and to reconstitute lysis by CTL A2 264. Two antigenic HPLC fractions obtained from the natural peptide extract had an almost identical retention time and conductivity as compared with those antigenic HPLC fractions derived from the synthetic p53.264–272 and p53.260–272 peptides. As 4-min HPLC fractions were collected at the relevant retention time, the peak of CTL activity occurred in almost identical HPLC fractions, although the retention time of the synthetic p53.264–272 and p53.260–272 peptides differed from each other by ~4 min. To separate either peptide from one another and identify the natural antigenic peptide, half of the pooled antigenic HPLC fractions of the HPLC-separated natural peptide extract (A) as well as 60 pmol of synthetic 264–272 (B) and 260–272 (C) peptides were used for rechromatography by R-P-HPLC. HPLC conditions remained almost the same, yet 0.5-min fractions (25 μl/fraction) were collected at the relevant retention time in order to discriminate between the 264–272 and the 260–272 peptides. 51Cr-labeled T2 target cells were pulsed for 40 min under serum-free conditions with individual HPLC fractions (fractions derived from the rechromatography of natural peptides: 18 μl; fractions 8, 19, 20, and 29 derived from synthetic p53 peptides: 2 μl; fractions 9–18 and 21–28 derived from synthetic p53 peptides: 0.02 μl, 0.2 μl, and 2 μl) and tested for susceptibility to lysis by CTL A2 264 at an E/T of 13:1 in a 6-h 51Cr-release assay. The HPLC profile (absorbance: — (A): first HPLC separation of natural peptide extracts and the specific lysis (shaded columns) of T2 targets sensitized with individual HPLC fractions obtained from the rechromatography of natural peptides (A), 2 μl (fractions 8, 19, 20, and 29), and 0.2 μl (fractions 9–18 and 21–28) of HPLC fractions obtained from the synthetic p53.264–272 (B) and p53.260–272 (C) peptides is shown.

Several recent studies have focused on the mutational alteration within a peptide epitope to demonstrate a way by which viruses can escape recognition by epitope-specific CTLs (46–50). This is generally believed to involve peptide binding to the MHC class I molecule, or recognition by the TCR (46–50). However, in a recent report it was shown that a mutation within a viral peptide epitope affected the production of precursor peptides by proteasomal degradation through the introduction of a dominant new proteasomal cleavage site within the viral epitope (31). Based on studies that evaluated the effect of synthetically altering residues...
flanking a peptide epitope on its processing, it would be predicted that point mutation outside of the epitope could also interfere with its presentation (30, 36, 44, 51–56). To our knowledge, our studies represent the first report of such a naturally occurring mutation outside the epitope that alters CTL recognition of a flanking peptide. In fact, the single mutation described here interferes not only with the proteasomal processing of putative precursor peptides, but also with the generation of the optimal CTL epitope itself. It is present unclear why the R to H mutation interferes with the COOH-terminal proteasomal cleavage. It is possible that a change in charge and size of the flanking residue influences the COOH-terminal cleavage site used by the proteasome. However, as the possibility that a particular site will be preferred for proteasomal cleavage is dependent on amino acid sequences within the epitope (31, 51), as well as on flanking residues both up- and downstream (30, 36, 44, 51–56), it is likely that the rules by which ligand generation is governed are more complex and not yet understood.

Treatment of cells with IFN-γ has been reported to alter the processing kinetics, quantity, and quality of MHC class I–bound peptide ligands by affecting the expression both of the IFN-γ-inducible proteasomal subunits (LMP2, LMP7, and M ECL-1) and the IFN-γ-inducible PA28α/β activator complex (11S regulator) of the 20S proteasome (28, 29, 41, 43, 57–69). Exposing Saos-2/273 (R to H) p53 transfectants to IFN-γ (10 ng/ml for 20 h) resulted in an only partial reconstitution of lysis by CTL A2 264 (20% specific lysis of nonpretreated Saos-2/273 cells versus 31% specific lysis of IFN-γ pretreated Saos-2/273 targets at an E/T of 20:1) (Häussler, A., and M. Theobald, unpublished observation). The molecular basis of this IFN-γ-mediated partial reconstitution of Saos-2/273 killing by CTL A2 264 on the level of both the proteasomal subunit composition (LMP2, LMP7, and M ECL-1) and the PA28 activator (11S regulator) expression is currently under investigation.

Based on these results, it may be concluded not only that the p53 mutation at residue 273 is associated with malignant transformation, but that it can also affect CTL recognition in vitro and in vivo of tumor cells carrying this mutation. It is tempting to speculate that cells harboring this mutation may have a competitive edge for growth in A*0201+ individuals by evading CTL recognition (70). Several other reports have shown that vaccination of mice with the intact p53 protein expressed in a viral vector or with p53 peptides pulsed onto dendritic cells can prevent growth in vivo of tumours expressing high levels of p53 (5, 7). Furthermore, recent studies have demonstrated the ability of p53.264–272 epitope–specific Hu CTL to lyse squamous cancer cells (6). Thus, knowledge not only of the antigenic epitopes of p53, but also of the biological role of their sequence context and its modulation by frequently arising mutations may explain disease progression, and may assist in the design of efficacious cancer vaccines.

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