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Splicing of Distant Peptide Fragments Occurs in the Proteasome by Transpeptidation and Produces the Spliced Antigenic Peptide Derived from Fibroblast Growth Factor-5

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Peptide splicing is a newly described mode of production of antigenic peptides presented by MHC class I molecules, whereby two noncontiguous fragments of the parental protein are joined together after excision of the intervening segment. Three spliced peptides have been described. In two cases, splicing involved the excision of a short intervening segment of 4 or 6 aa and was shown to occur in the proteasome by transpeptidation resulting from the nucleophilic attack of an acyl-enzyme intermediate by the N terminus of the other peptide fragment. For the third peptide, which is derived from fibroblast growth factor-5 (FGF-5), the splicing mechanism remains unknown. In this case, the intervening segment is 40 aa long. This much greater length made the transpeptidation model more difficult to envision. Therefore, we evaluated the role of the proteasome in the splicing of this peptide. We observed that the spliced FGF-5 peptide was produced in vitro after incubation of proteasomes with a 49-aa-long precursor peptide. We evaluated the catalytic mechanism by incubating proteasomes with various precursor peptides. The results confirmed the transpeptidation model of splicing. By transfecting a series of mutant FGF-5 constructs, we observed that reducing the length of the intervening segment increased the production of the spliced peptide, as predicted by the transpeptidation model. Finally, we observed that trans-splicing (i.e., splicing of fragments from two distinct proteins) can occur in the cell, but with a much lower efficacy than splicing of fragments from the same protein. The Journal of Immunology, 2010, 184: 3016–3024.

Antigens presented by MHC class I molecules are composed of peptides of 8–11 aa in length and are recognized at the cell surface by specific CD8+ T lymphocytes. The presentation of these antigenic peptides by infected cells or tumor cells allows their specific recognition and lysis by T lymphocytes (1). These peptides are usually derived from the degradation of intracellular proteins by the proteasome. The 26S proteasome consists of a 20S core cylindrical complex capped at both ends by 19S regulatory particles (2). Catalytic activity is exerted by the 20S proteasome composed of four stacked rings of seven α- or β-subunits each (3). 19S regulatory complexes bind the α-rings of the 20S core and are responsible for substrate capture and translocation of these substrates into the 20S catalytic complex. Enzymatic activities are mediated by three of the seven β-subunits of the 20S proteasome, β1, β2, and β5, corresponding to three major activities, caspase-like, trypsin-like, and chymotrypsin-like, respectively (4, 5). The 20S proteasome is classified as a N-terminal nucleophile hydrolase, with the N-terminal threonine of the active β-subunits acting as the nucleophile catalyst (3, 6, 7).

Most MHC class I–associated antigenic peptides correspond to small continuous fragments of intracellular proteins. However, in the past few years, some antigenic peptides were found to be created by the splicing of two noncontiguous fragments of the respective parental proteins (Fig. 1A). The first example is a 9-aa peptide derived from fibroblast growth factor-5 (FGF-5) and made up of two fragments of five and four residues, which are located 40 aa apart in the parental protein (8). Another is a nonamer peptide produced from the melanomasomal protein gp100 by excision of 4 aa and splicing of the two flanking fragments: one of three residues and one of six residues (9). The third example of spliced peptide is a human minor histocompatibility Ag created by a polymorphism in the SP110 gene (10). This epitope is made of two noncontiguous fragments of four and six residues, separated by 6 aa in the SP110 protein. In this case, the two fragments are spliced together, but in the reverse order to that in which they appear in the parental protein.

The splicing mechanism was studied in detail for the gp100 and SP110 peptides (9, 10). In both cases, quite surprisingly, the proteasome was found to produce the spliced peptide, and the splicing reaction could be reproduced in vitro by incubating precursor peptides with purified proteasomes. The splicing process involves a first step in which the intervening fragment is cleaved off. The N-terminal fragment of the final epitope is retained on the catalytic subunit as an acyl-enzyme intermediate through an ester bond with the side chain of the catalytically active threonine of one of the β-subunits (Fig. 1B). This ester bond then undergoes a nucleophilic attack by the free N terminus of the other peptide fragment, which results in a transpeptidation reaction forming a new peptide bond. In this reaction, the peptide fragment competes for this nucleophilic attack with water molecules, which, during normal proteolysis, hydrolyze the ester bond of the acyl-enzyme intermediate. Transpeptidation is presumably favored...
within the catalytic chamber of the proteasome because of its confined space, which prevents rapid diffusion of the peptide fragments and increases their relative concentration (9, 11).

In the case of the FGF-5 spliced peptide, the mechanism of splicing has not been worked out. Hanada et al. (8) showed that recognition of tumor cells by the cognate CTL was reduced after treatment with proteasome inhibitor lactacystin or after TAP inhibition with ICP47. These results suggested that the proteasome was also involved in the production of this spliced peptide. However, the length of the intervening fragment (40 aa) to remove before splicing of the two flanking fragments made the transpeptidation model inside the proteasome more difficult to envision, because it implies that the two distinct fragments remain within the catalytic chamber of the proteasome during the time needed to cleave off the long intervening segment. Therefore, we set out to confirm the role of the proteasome in the splicing of the FGF-5 peptide. This also led us to evaluate the influence of the length of the intervening segment on the efficiency of splicing and to test the possibility of trans-splicing between two distinct proteins.

Materials and Methods

Cell lines and CTL clones

Autologous renal cell carcinoma line 1764-RCC and COS-7 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% FCS. All culture media were supplemented with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml).

CTL C2 is a human CTL clone that recognizes the FGF-5 spliced peptide NTY A-RTK in 0.4-cm cuvettes, using a Gene-omnium 400 µl water. One half of each 20S proteasomes in 20 µl Tris 10 mM (pH 7.5) was mixed with 100 µM 20S proteasomes in the same buffer. Digestions were stopped by the addition of 200 µl of lactacystin. Cells were washed, and one quarter (Fig. 7A) or one quarter (Fig. 7B) of each digest was diluted in 100 µl of each precursor peptide with 1 µM lactacystin. Cells were incubated with 25,000 cells CTL C2 per well in 100 µl medium. One quarter of each of 20S proteasomes were purified from human erythrocytes, as previously described (15). Digestion of the FGF-5 49-mer precursor peptide (Fig. 3A) was performed by incubating 8 µg peptide with 1 µM 20S proteasomes in 20 µl Tris 10 mM (pH 7.5) for 4 h. After washing, half of the cells were loaded with the antigenic peptide (2 µg/ml) for 4 h. For the CTL assay, 25,000 cells per well (96-well plate) were incubated with 20,000 CTL C2. In Fig. 6B, COS-7 cells stably transfected with HLA-A3 were electroporated with 50 µg of each peptide using the same parameters as above. Cells were diluted to a peptide concentration of 3.3 µM and incubated for 4 h. After washing, half of the cells were loaded with the antigenic peptide (2 µg/ml) for 30 min. For the CTL assay, 40,000 electroporated cells/well were incubated with 20,000 cells CTL C2. In Fig. 7B, COS-7 cells stably transfected with HLA-A3 were electroporated with 70 µg of each peptide. Cells were then diluted to a peptide concentration of 4.7 µM and incubated for 4 h in culture medium with or without 1 µM lactacystin. Cells were washed, and half of them were loaded with antigenic peptide (2 µg/ml) for 1 h. For the CTL assay, 25,000 cells per well (96-well plate) were incubated with 20,000 CTL C2. In Fig. 6B, COS-7 cells stably transfected with HLA-A3 were electroporated with 50 µg of each peptide using the same parameters as above. Cells were diluted to a peptide concentration of 3.3 µM and incubated for 4 h. After washing, half of the cells were loaded with the antigenic peptide (2 µg/ml) for 30 min. For the CTL assay, 40,000 electroporated cells/well were incubated with 20,000 cells CTL C2. In Fig. 7B, COS-7 cells stably transfected with HLA-A3 were electroporated with 70 µg of each peptide. Cells were then diluted to a peptide concentration of 4.7 µM and incubated for 4 h. After washing, half of the cells were loaded with the antigenic peptide (2 µg/ml) for 30 min. For the CTL assay, 40,000 electroporated cells/well were incubated with 25,000 cells CTL clone 14 (9). The production of IFN-γ was measured by ELISA after an overnight incubation.

Peptide electroporation followed by CTL assay

In Fig. 2B, HEK293-EBNA cells were treated for 1 h with the indicated amounts of lactacystin. Cells were then washed with PBS and electroporated in 400 µl electroporation buffer (K2HPO4/KH2PO4 [10 mM; pH 7.5], MgCl2 [1 mM], and sucrose [250 mM]) with 100 µM 30-mer peptide NTYA-SAILHKRGCSPRVKFQFHISTHLPFRK in 0.4-cm cuvettes, using a Gene-pulser Xcell electroporator (Bio-Rad, Hercules, CA) functioning in square wave mode with 10 pulses of 0.1 ms at 480 V separated by 1-s intervals. Cells were diluted to a peptide concentration of 6.7 µM and incubated for 4 h in culture medium with or without 1 µM lactacystin. Cells were washed, and half of them were loaded with antigenic peptide (2 µg/ml) for 1 h. For the CTL assay, 25,000 cells per well (96-well plate) were incubated with 20,000 CTL C2. In Fig. 6B, COS-7 cells stably transfected with HLA-A3 were electroporated with 50 µg of each peptide using the same parameters as above. Cells were diluted to a peptide concentration of 3.3 µM and incubated for 4 h. After washing, half of the cells were loaded with the antigenic peptide (2 µg/ml) for 30 min. For the CTL assay, 40,000 electroporated cells/well were incubated with 20,000 cells CTL C2. In Fig. 7B, COS-7 cells stably transfected with HLA-A3 were electroporated with 70 µg of each peptide. Cells were then diluted to a peptide concentration of 4.7 µM and incubated for 4 h. After washing, half of the cells were loaded with the antigenic peptide (2 µg/ml) for 30 min. For the CTL assay, 40,000 electroporated cells/well were incubated with 25,000 cells CTL clone 14 (9). The production of IFN-γ was measured by ELISA after an overnight incubation.

Proteasome digestions of synthetic peptides and CTL assay of the digests

Synthetic peptides were produced using Fmoc solid-phase chemistry. Peptides were dissolved in DMSO at 20 mg/ml and stored at −20°C. 20S standard proteasomes were purified from human erythrocytes, as previously described (15). Digestion of the FGF-5 49-mer precursor peptide (Fig. 3A) was performed by incubating 8 µg peptide with 1 µg 20S proteasomes in 20 µl Tris 10 mM (pH 7.5) for 37°C. In Fig. 4B, 4 µg each of the combined peptides were mixed with 1 µg 20S proteasomes in 20 µl Tris 10 mM (pH 8). In Fig. 6A, we incubated 7 µg of each precursor peptide with 1 µg 20S proteasomes in Tris 10 mM (pH 8). In Fig. 7A, 2 µg of each precursor peptide were incubated with 1 µg 20S proteasomes in the same buffer. Digestions were stopped by the addition of 2 µl 10% trifluoroacetic acid, lyophilized, and resuspended in 20 µl water. One half (Fig. 6A) or one quarter (Fig. 7A) of each digest was diluted in 100 µl
plasmids generated were verified by sequencing with an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Transient transfection followed by CTL assay**

HEK293-EBNA cells (25,000 cells/well), which are HLA-A3+ were plated in flat-bottom 96-well microplates 48 h before transfection. Cells were transfected using Lipofectamine reagent (Invitrogen), with the indicated amounts of pEF6 plasmid coding for the FGF-5 genes described above. Twenty-four hours after transfection, the medium was carefully removed, and 25,000 cells CTL C2 were added in X-vivo 10 medium. After an overnight incubation, the supernatants were collected, and their IFN-γ was measured 18 h later.

**FIGURE 2.** Proteasome inhibition prevents the production of the spliced FGF-5 antigenic peptide. A, Autologous tumor cells 1764-RCC were treated with the indicated proteasome inhibitors after acid elution of MHC-bound peptides and then were tested for recognition by CTL clone C2. The production of IFN-γ was measured after an overnight incubation. Where indicated, cells were loaded with synthetic peptide NTYASPRFK before the CTL assay to check their Ag-presentation capacity. Relative activities (left panel) were calculated first as a proportion of the activity measured with the synthetic peptide control. This proportion was then related to that observed without treatment, which was taken as 100%. Actual values are shown in the right panel. Error bars show standard deviations of triplicates. B, Lactacystin-treated HEK293-EBNA cells were electroporated with a 30-mer precursor peptide (NTYASAIBKRGCSVRKPQHITHFPRFK) and tested for recognition by CTL C2. HEK293-EBNA cells naturally express HLA-A3. Where indicated, synthetic peptide NTYASPRFK was loaded 1 h before the CTL assay. The production of IFN-γ was measured after an overnight incubation. Relative activities (left panel) were calculated as above. Actual values are shown in the right panel. Error bars show standard deviations of triplicates.

**FIGURE 3.** In vitro production of the spliced FGF-5 antigenic peptide by proteasomes. A, CTL recognition of digests obtained by in vitro incubation of the indicated FGF-5 49-mer precursor peptide with purified 20S standard proteasomes. Reactions were stopped at 0, 60, 120, or 240 min. Digests were then loaded onto HLA-A3+ EBV-B cells before the addition of CTLs. Production of IFN-γ was measured 18 h later. B, MS/MS fragmentation spectrum of the doubly charged ion with m/z 5422+ observed in the 240-min digest shown in A and MS/MS fragmentation spectrum of the doubly charged ion with m/z 5422+ of the synthetic peptide NTYASPRFK. The fragment ions that were detected are indicated above the peptide sequence for N-terminal b ions and below for C-terminal y ions. Ion m/z 5332+ is a dehydrated form of ion m/z 5422+.

X-vivo 10 medium and pulsed in duplicate wells containing 20,000 EBV-B cells expressing the appropriate HLA molecule. CTLs (20,000/well) were added after 30 min of incubation, and the production of IFN-γ in supernatants was measured by ELISA after an overnight coculture.

**Generation and analysis of mutated plasmid constructs**

FGF-5 constructs with internal deletions were generated from a plasmid encoding the full-length FGF-5 gene by carrying out a long PCR with one primer, using QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, LA Jolla, CA). For each deletion construct, we designed a primer composed ≥20 nucleotides of the FGF-5 sequence situated on each side of the deletion. To generate the FGF-5 Δ1 construct, encoding the deletion of aa 192–201, we used the primer 5′-GGGCGGGATGTTGATGTGTGGCCTG- CAGCCCCGGGTTAAACC-3′. For the FGF-5 Δ2 construct, encoding the deletion of aa 187–206, we used the primer 5′-AGAAGCTGAAAAAAC- CAGGGCGGGAAACCCCAGCATATCTCTACCC-3′; for FGF-5 Δ3, encoding the deletion of aa 181–212, we used the primer 5′-CTCTATGCGC- TCAGCAATACATAGAACCCATTTTCTGCCAAGATTCAAGC-3′. Then, we amplified, by PCR using a Taq polymerase, the entire FGF-5 coding region containing the internal deletion and cloned the PCR products into pEF6/V5-His TOPO (Invitrogen). Point mutations in the FGF-5 and gp100 sequences were also introduced by carrying out PCR with the QuickChange Multi Site-Directed Mutagenesis Kit. We used a pC DNA3 plasmid containing the wild-type (wt) gp100 cDNA as a template and specific primers for each mutation required. For FGF-5, the template was plasmid pEF6, containing the full-length FGF-5 cDNA. The sequences of the coding region of all of the genes described above were verified with an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

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**Transient transfection followed by CTL assay**

HEK293-EBNA cells (25,000 cells/well), which are HLA-A3+, were plated in flat-bottom 96-well microplates 48 h before transfection. Cells were transfected using Lipofectamine reagent (Invitrogen), with the indicated amounts of pEF6 plasmid coding for the FGF-5 genes described above. Twenty-four hours after transfection, the medium was carefully removed, and 25,000 cells CTL C2 were added in X-vivo 10 medium. After an overnight incubation, the supernatants were collected, and their IFN-γ content was measured by ELISA.

**COS-7 cells (15,000 cells/well) were plated in flat-bottom 96-well microplates 24 h before transfection. Cells were transfected using Lipofectamine, with 50 ng plasmid DNA coding for the appropriate HLA molecule (HLA-A3, -A32, or -A2) and 200 ng plasmid DNA of FGF-5 or
gn100 plasmid construct (empty vector was added when required to transfect an equal amount of DNA in all conditions). CTL C2 (Fig. 6C), CTL 14, or CTL 7 (Fig. 7C) was added 24 h later in X-vivo 10 medium, and the supernatant was collected after 20 h of coculture to estimate its IFN-γ content.

**Transient transfection of FGF-5 followed by kinetics of intracellular clearance and Western blots**

HEK293-EBNA cells (3.10⁶ cells/flask) were plated in T75 flasks 24 h before transfection. Cells were transfected using TransIT-LT1 reagent (Mirus, Madison, WI) with 20 µg pEF6 plasmids and using the FGF-5 constructs described above. Twenty-four hours after transfection, cells were detached with PBS, and dry pellets were frozen immediately. Cells were further lysed on ice in Cytobuster buffer (Thermo Scientific) and treated with 100 µl protease inhibitor mixture (Complete, Roche Applied Science, Indianapolis, IN). Each sample was sonicated for 10–15 s and centrifuged at 20,000 g for 25 min at 4˚C. Protein concentration in supernatants was determined with the BCA Protein Assay (Pierce, Rockford, IL). Twenty-five micrograms of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with primary Abs (anti–FGF-5: sc-1362, purchased from Cell Signaling Technology [Beverly, MA] or anti–β-actin: AC-15, purchased from Sigma-Aldrich) and horseradish peroxidase-labeled secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

**HPLC and mass spectrometry**

Mass spectrometry (MS) was performed on-line with an LCQ Deca XP Plus ion-trap mass spectrometer equipped with an electrospray ionization source (ThermoFinnigan, San Jose, CA). Mass spectra were acquired in a mode that alternated single MS scans (m/z 200–2000) with tandem MS (MS/MS) scans. Samples were separated by reverse-phase chromatography on a PepMap C18 0.3x15 column (LC Packings, Sunnyvale, CA) and eluted using a 55-min linear gradient of acetonitrile in water (5–50%) containing 0.05% trifluoroacetic acid with a flow rate of 4 µl/min obtained by splitting.

**Results**

We first confirmed the involvement of the proteasome in the processing of the FGF-5 antigenic peptide (Fig. 1) by evaluating the recognition of cells treated with proteasome inhibitor lactacystin or epoxomicin by FGF-5–specific CTL clone C2 (Fig. 2). We observed a reduced recognition of treated cells with autologous tumor cells (Fig. 2A), as well as with HEK293-EBNA cells electroporated with a precursor peptide encompassing the FGF-5 spliced peptide (Fig. 2B).

To directly evaluate the ability of the proteasome to produce the FGF-5 spliced peptide, we incubated purified 20S proteasomes with a 49-mer precursor peptide encompassing the complete sequence of FGF-5 between the two spliced fragments NTYAS and PRFK. We then examined recognition by CTL C2 of target cells loaded with the digests. The digests were clearly able to activate the CTL, and this ability increased with digestion time (Fig. 3A). The presence of the spliced antigenic peptide was evaluated by HPLC combined with MS/MS in a digest obtained after 240 min of incubation of the 49-mer precursor peptide with 20S proteasomes. The antigenic peptide was observed in this digest and was detected as a doubly charged ion with m/z 542.2+. Its retention time and fragmentation pattern were identical to those of the synthetic peptide NTYASPRFK (Fig. 3B).

The efficiency of the splicing reaction was particularly low; a comparison of the level of CTL activation obtained with the digests or with a titration curve of the synthetic peptide provided a rough estimate of one spliced peptide produced from ~5 x 10⁵ molecules of precursor peptide.

We also used MS to estimate the degradation of the precursor peptide in those digestions. We observed a biphasic relationship between production of the spliced peptide and precursor degradation, with a first linear phase likely corresponding to direct production of the spliced peptide from the long precursor, and a second phase during which spliced peptide production kept increasing, despite complete degradation of the precursor (Supplemental Fig. 1A). The latter phase probably resulted from re-entry and further processing of degradation intermediates, a phenomenon favored by in vitro digestion conditions involving a single substrate. In line with this interpretation, in those digests we observed an identical biphasic production of the fragments to be spliced: NTYAS and PRFK (Supplemental Fig. 1B).

If the FGF-5 peptide is produced in the proteasome by the same splicing mechanism as the two other spliced peptides, NTYAS is the fragment that would be involved in the acyl-enzyme intermediate, whereas fragment PRFK would be responsible for the nucleophilic attack of this intermediate to form the spliced peptide NTYASPRFK (Fig. 4A). The formation of the acyl-enzyme

**FIGURE 4.** Mechanism of production of the spliced FGF-5 antigenic peptide. A. Model of the catalytic mechanism of the splicing reaction occurring within the proteasome to produce the FGF-5 spliced peptide. The balls represent the catalytically active β-subunits of the proteasome with the hydroxyl group of the side chain of the N-terminal threonine. B. CTL recognition of digests obtained by in vitro incubation of 20S proteasomes with various synthetic peptides combined in a pairwise manner. Digests obtained by incubating proteasomes with the indicated peptides were loaded onto HLA-A3+ EBV-B cells before the addition of CTLs. Production of IFN-γ was measured 18 h later. Ac-PRFK, N-terminally acetylated peptide with sequence PRFK.

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intermediate would be coupled to the cleavage of the peptide bond C-terminal from NTYAS in the parental polypeptide. We investigated the requirement for cleavage of this peptide bond by incubating 20S proteasomes with various synthetic peptides combined in a pairwise manner. Digests obtained with peptides NTYASAIH and PRFK were strongly recognized by the CTLs, whereas digests obtained with peptides NTYAS and PRFK were not (Fig. 4B). This indicated that the formation of an acyl-enzyme intermediate linking NTYAS to the proteasome is required for the splicing reaction. This experiment also confirmed that the energy of the new peptide bond created is provided by the cleavage of the bond between residues S and A of fragment NTYASAIH. We then observed that production of the spliced peptide was abolished when the amino terminal group of fragment PRFK was blocked by acetylation (Fig. 4B). This result confirmed that this amino group was responsible for the nucleophilic attack of the acyl-enzyme intermediate and, thereby, supports the transpeptidation model of splicing leading to the final antigenic peptide.

A major difference between the splicing of the FGF-5 peptide and the splicing of the gp100 and SP110 peptides studied before is the much longer length of the intervening sequence: 40 aa instead of 4 and 6 aa. To evaluate the effect of the length of the intervening sequence, we constructed three plasmids encoding mutated FGF-5 with shortened intervening sequences of different lengths. These mutated FGF-5, called Δ1, Δ2, and Δ3, consisted of an intervening sequence of 30, 20, and 8 residues, respectively (Fig. 5A). Amino acids were deleted in the middle of the intervening sequence to maintain the residues flanking the two fragments of the final epitope and to minimize potential cleavage modifications at these sites. Increasing amounts of these plasmids were then transfected into HEK293-EBNA cells, and the production of the spliced FGF-5 peptide was evaluated by measuring the ability of transfected cells to activate CTL C2. The efficiency of production of the spliced peptide clearly increased when the size of the intervening sequence was reduced (Fig. 5B). The greatest CTL activation was observed with cells transfected with the construct encoding an intervening sequence of 8 aa, whereas decreased activation was observed with cells transfected with the wt construct encoding a 40-aa intervening segment. The result obtained with the mutant encoding the 30-aa segment was similar to that obtained with the wt construct. We considered the fact that the better production of the peptide from the short mutants might result from a misfolding of the protein, which could increase its degradation by the proteasome. To exclude this possibility, we compared the degradation of the four different FGF-5 proteins in transfected cells. We performed Western blots on transfected cells treated with cycloheximide, an inhibitor of protein synthesis, using an Ab directed against the C terminus of FGF-5. This Ab detects three specific bands, presumably corresponding to three glycosylated forms of FGF-5 (Fig. 5C) (16). The proteins encoded by the different mutants disappeared at a similar rate. Surprisingly, the wt FGF-5 disappeared faster than the mutants. Brefeldin A, which blocks the transport of secretory proteins through the Golgi apparatus, moderately reduced the degradation of the FGF-5 proteins encoded by the different constructs. This excluded a major impact of secretion and confirmed that intracellular degradation was primarily responsible for the disappearance of the protein. Although the reason for the faster degradation of the wild-type protein is unclear, the similar degradation of the various mutant proteins supports the conclusion that the better production of the spliced peptide from the short mutant proteins is due to the shorter length of the intervening segment favoring the splicing reaction.

Because splicing can occur between fragments that are 40 aa apart in the parental protein, we asked whether splicing could also occur between fragments located in distinct parental proteins. Such trans-splicing is conceivable in theory, because it was shown that...
the inner channel of the proteasome, with a diameter of 13 Å (17), can accommodate more than one polypeptide chain (18, 19). The proteasome was also able to bind and process two substrates simultaneously: one in each catalytic chamber (20). To test this hypothesis, we designed plasmids encoding full-length FGF-5 proteins containing a point mutation in fragment NTYAS or PRFK. The mutations were chosen to abolish the recognition of the corresponding spliced peptide by CTL C2 (8). They were located within the fragments to minimize cleavage site changes. The first mutant (FGF-5 Y174A) coded for alanine instead of tyrosine in fragment NTYAS; the other mutant coded for alanine instead of arginine in fragment PRFK (FGF-5 R218A). Upon cotransfection of both mutants, the only way to produce the spliced antigenic peptide would be through splicing of fragments NTYAS and PRFK from two distinct parental proteins. Before testing whether splicing could occur between these two mutated proteins, we verified that the proteasome was able to produce the spliced antigenic peptide in vitro, by incubating the corresponding mutated precursor peptides with purified 20S proteasomes. The digests obtained were pulsed onto HLA-A3+ EBV-B cells and tested for CTL recognition. As expected, incubations of each mutated precursor alone with 20S proteasomes did not lead to any CTL activation (Fig. 6A). However, when these two mutated precursors were incubated simultaneously with 20S proteasomes, the corresponding digests were able to activate CTL C2. These results indicated that the FGF-5 spliced peptide can be efficiently produced in vitro from these two different mutated precursor peptides. Splicing from distinct peptides also occurs in the cell; we obtained similar results when we introduced these mutated precursor peptides into COS-A3 cells by electroporation (Fig. 6B). To determine whether splicing can occur in vivo between fragments of two proteins, the mutated plasmids FGF-5 Y174A and FGF-5 R218A were cotransfected in COS cells, together with a plasmid coding for HLA-A3. As shown in Fig. 6C, the transfection of wt FGF-5 led to strong activation of CTL C2, whereas the transfection of each mutated construct did not activate the CTL, as expected. When the two mutated FGF-5 constructs were transfected together, a very weak activation of CTL C2 was detected. Although this activation was significantly greater than the background, it was ~30-fold lower than the one triggered by transfection of wt FGF-5. We concluded that trans-splicing of the FGF-5 peptide from two distinct protein substrates can occur but with a very low efficiency compared with splicing from a single protein substrate.

Using the same approach, we also tested the efficacy of trans-splicing in the case of the spliced gp100 peptide RTKQLYPEW presented by HLA-A32 (9) (Fig. 1). We designed plasmids encoding full-length gp100 proteins containing a point mutation in fragment

**FIGURE 6.** Assay for trans-splicing from two distinct peptides or proteins for the spliced FGF-5 peptide. A. CTL recognition of digests obtained by in vitro incubation of 20S proteasomes with the indicated precursor peptides of the spliced antigenic peptide NTYASPRFK. Reaction was stopped after 120, 240, or 360 min of digestion. The digests were then loaded on HLA-A3+ EBV-B cells before the addition of CTL C2. B. COS-7 cells stably transfected with HLA-A3 were electroporated with the indicated precursor peptides and tested for recognition by CTL C2. Where indicated, cells were also loaded with exogenous peptide NTYASPRFK to control cell viability. Error bars show standard deviations of triplicates. C. COS-7 cells were transiently transfected with wt or mutated full-length FGF-5 cDNA constructs, together with an HLA-A3 cDNA construct. Transfected cells were then tested for recognition by CTL C2. Where indicated, cells were also loaded with exogenous peptide NTYASPRFK. Error bars show standard deviations of triplicates.
RTK or QLYPEW so that CTL recognition of the corresponding spliced peptides was abolished. The first mutant (gp100 T41A) coded for alanine instead of threonine in fragment RTK, and the other mutant coded for alanine instead of glutamate in fragment QLYPEW (gp100 E51A). Again, we verified that the proteasome was able to produce the spliced antigenic peptide in vitro by incubating the corresponding mutated precursor peptides RAKAWNQRQLYPEW and RTKAWNQRQLYPAW with purified 20S proteasomes. The digests obtained were pulsed onto HLA-A32 EBV-B cells and tested for CTL recognition. As expected, incubations of each mutated precursor alone with 20S proteasomes did not lead to significant CTL activation (Fig. 7A). However, when these two mutated precursors were incubated simultaneously with 20S proteasomes, the corresponding digests were able to activate CTL 14. Thus, the spliced peptide RTKQLYPEW can be efficiently produced in vitro from these two different mutated precursor peptides. Splicing from distinct peptides was also observed in vivo after electroporation in the cell (Fig. 7B). Finally, to determine whether trans-splicing can occur in vivo between fragments of two gp100 proteins, mutated plasmids gp100 T41A and gp100 E51A were co-transfected in COS cells, together with a plasmid coding for HLA-A32. As shown in Fig. 7C (left panel), transfection of wt gp100 led to strong activation of CTL 14. However, no CTL activation was detected after cotransfection of the two mutated gp100 T41A and gp100 E51A. The mutant constructs were functional, because they encoded another peptide derived from a different region of gp100 equally as well as the wt gp100 (Fig. 7C, right panel). Similar results were obtained by transfecting HEK293-EBNA cells or by using another pair of mutant gp100 constructs encoding RTA and QLYPEA (data not shown). We concluded that trans-splicing of fragments from distinct gp100 proteins does not occur in vivo at a level that is sufficient for recognition of the spliced peptide by our CTL.

Discussion

By confirming the direct role of the proteasome in the production of the spliced antigenic peptide derived from FGF-5, our results further strengthen the notion that peptide splicing by the proteasome contributes to the diversity of the peptide repertoire presented by MHC class I molecules. We also confirm the catalytic mechanism we proposed for the two other spliced antigenic peptides, which is

![FIGURE 7. Assay for trans-splicing from two distinct peptides or proteins for the spliced gp100 peptide. A, CTL recognition of digests obtained by in vitro incubation of 20S proteasomes with wt or mutated precursor peptides of the spliced antigenic peptide gp10040–42/47–52 RTKQLYPEW. Reaction was stopped after 60 or 120 min of digestion. The digests were then loaded on HLA-A32 EBV-B cells before the addition of CTL clone 14, which recognizes gp100 peptide RTKQLYPEW. B, COS-7 cells stably transfected with HLA-A32 were electroporated with the indicated precursor peptides and tested for recognition by CTL 14. Where indicated, cells were also loaded with exogenous peptide RTKQLYPEW to control cell viability. Error bars show standard deviations of triplicates. C, COS-7 cells were transiently transfected with wt or mutated full-length gp100 cDNA constructs, together with an HLA-A32 (left panel) or an HLA-A2 cDNA construct (right panel). Transfected cells were then tested for recognition by CTL clone 14 (left panel; spliced peptide gp10040–42/47–52 RTKQLYPEW) or CTL 7, which recognizes peptide gp100209–217 ITDQVPFSV presented by HLA-A2 (right panel). Where indicated, cells were also loaded with exogenous peptide RTKQLYPEW (left panel). Error bars show standard deviations of triplicates.](http://www.jimmunol.org/)


a transpeptidation occurring at the active N-terminal threonine of a β-catalytic subunit of the proteasome. During cleavage of the peptide bond, the hydroxyl group of the side chain of this threonine makes an ester bond with the C terminus of the N-terminal peptide fragment produced by the cleavage, which then remains transiently linked to the proteasome subunit as an acyl-enzyme intermediate. During normal proteolysis, this acyl-enzyme intermediate is rapidly hydrolyzed, and the fragment is liberated. In the splicing reaction, the N terminus of another peptide fragment competes with water molecules and occasionally performs a nucleophilic attack of the acyl-enzyme intermediate, resulting in a transpeptidation reaction that produces the final antigenic peptide. One implication of this mechanism is that it is not determined by a particular sequence motif but could occur at any major cleavage site by the proteasome, a prediction that we previously confirmed in the model of the gp100 spliced peptide (9).

Another prediction of this catalytic model is that the efficiency of splicing depends on the power with which the free peptide fragment competes with water molecules for the nucleophilic attack of the acyl-enzyme intermediate. Several factors need to be considered. One is imposed by the structure of the catalytic chamber of the proteasome, which is confined and prevents rapid diffusion of the peptide fragments liberated by the action of the catalytic subunits. The relative concentration of these fragments is significantly increased, favoring transpeptidation over hydrolysis.

A second factor to consider in evaluating the efficiency of production of a given spliced peptide is the probability of interaction of the two relevant fragments. This probability is greater for peptide fragments that are produced simultaneously in the catalytic chamber. It is also dependent on the half-life of the acyl-enzyme intermediate (9, 21). Therefore, the probability of transpeptidation should be greater for peptide fragments that are not too distant in the parental protein, because they are more likely to be produced simultaneously during degradation of the protein, which is considered to occur mostly in a processive manner (22). However, we described in this paper efficient splicing between FGF-5 fragments that are located 40 aa apart in the parental protein. We observed, as expected, that reducing the length of this intervening segment increased the splicing efficiency. The observed splicing from the wt FGF-5 seems to imply that the acyl-enzyme intermediate NTYAS-proteasome is particularly stable and can survive the time required for production of fragment PRFK. As suggested by Borissenko and Groll, this could result from a high affinity of fragment NTYAS for the non-primed substrate-binding channel, which accommodates the peptide sequence at the N-terminal side of the cleavage site (Fig. 4) (21, 23). It can also depend on the affinity of fragment AIHRTE for the primed substrate-binding channel, which accommodates the peptide sequence at the C-terminal side of the cleavage site, because this fragment most likely needs to detach from this channel for fragment PRFK or H2O2 to come in and initiate the nucleophilic attack. However, depending on the sense of processive degradation of FGF-5, it is also possible that PRFK is produced first and maintained within the catalytic chamber until the acyl-enzyme intermediate involving NTYAS is formed.

A third factor is the nucleophilicity of the attacking peptide fragment. For FGF-5, the N terminus of the attacking fragment is the secondary amine of the proline. Compared with the primary amine of other residues, the N terminus of proline seems to be less reactive for nucleophilic attack as the result of a balance between the electron-releasing inducing effects and the steric effects of the cycle (24) and because of a higher pKa, resulting in more protonated forms at physiological pH. Yet, the proline N terminus is still substantially more nucleophilic than water molecules. It was proposed that efficient splicing is dependent on a proper positioning of the attacking fragment in the primed substrate-binding channel (23). Such positioning of the attacking peptide may allow the activation of its N terminus by a basic amino group present in the catalytic pocket, which may increase its nucleophilicity by attracting a proton, as described for the activation of the water molecule during hydrolysis of the acyl-enzyme intermediate (4). It is possible that fragment PRFK has a particularly high affinity for the primed substrate-binding channel of the proteasome and that this affinity contributes to explain the splicing efficiency of the FGF-5 peptide, despite the distance between the fragments.

We also examined the possibility of peptide splicing between two distinct substrates in living cells. Using synthetic peptides as substrates, which were digested in vitro with purified proteasomes (Figs. 4B, 6A, 7A, Supplemental Fig. 1A) or electroporated in living cells (Figs. 6B, 7B), we observed that the proteasome was intrinsically capable of transpeptidation between distinct substrates. In these experiments, the trans-splicing observed between distinct peptides was almost as efficient as the splicing from a single substrate. However, using cells expressing pairs of full-length mutated proteins as substrates, we detected a very weak production of the FGF-5 spliced peptide (Fig. 6C) and no production of the gp100 spliced peptide (Fig. 7C). The level of CTL activation obtained with cells transfected with the two FGF-5 mutants together was ∼30-fold lower than with the wt FGF-5. These results indicate that trans-splicing between distinct proteins is far less efficient than splicing from a single protein substrate and is unlikely to contribute significantly to the natural production of the FGF-5 spliced antigenic peptide. This might result from the fact that, among the numerous proteins being degraded by the proteasome in the cell, the chance of having precisely the two relevant proteins processed together in the same proteasome particle is very low. Additionally, for reasons of steric hindrance, it might be more difficult for two distinct proteins to fit together within a given proteasome than it is for two peptides. This might explain why in vivo trans-splicing between electroporated peptides is more efficient than in vivo trans-splicing between full-length proteins. If and when two relevant proteins are processed together, the intrinsically low splicing efficiency seems to result in the production of the relevant spliced peptide at a level that remains below or just above the threshold for CTL recognition. We conclude that most spliced antigenic peptides are expected to contain fragments of the same parental protein, but these fragments can be distant from each other in the parental protein.

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Disclosures

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References


Figure S1. In vitro production of the spliced FGF-5 antigenic peptide by proteasomes.

(A) Twenty micrograms of 49-mer precursor peptide were incubated with 2 µg of purified 20S standard proteasomes in 40 µl of Tris 10 mM pH 8 at 37°C. The left panel displays the CTL recognition of digests obtained after the indicated incubation times. The right panel shows the same results in relation to the degradation of the precursor peptide, which was determined by MS. In this experiment, the precursor peptide used had the cysteine in position 202 substituted with a serine to prevent dimerization and facilitate its quantification by MS.

(B) Production of fragments NTYAS (left panel) and PRFK (right panel) during the digestion shown in (A). The abundance of each fragment was determined by MS and is represented in relation to precursor peptide degradation determined in (A).