Antigenicity of Fusion Proteins from Sarcoma-associated Chromosomal Translocations

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INTRODUCTION

Neoplastic transformation occurs, in part, from the inactivation of tumor suppressor genes and/or the activation of proto-oncogenes. In some cases, the latter occurs as a result of chromosomal abnormalities such as translocations or inversions. Mechanistic theories of chromosomal translocations have been reviewed extensively (1–5). One mechanism involves a break within the coding sequence of each constituent gene, which generates a functional chimeric gene. This occurs primarily in sarcomas and leukemias, and the affected genes often encode transcription factors, suggesting a role in the transformation process. The fusion product retains the DNA binding specificity of one gene while inappropriately activating or repressing transcription through the transactivation domain of the other gene. This phenomenon is exclusive to the tumor cell and thus provides a tumor-specific marker.

Among solid tumors, SS, CCS, and DSRCT are connective tissue-related malignancies that primarily affect adolescents and young adults. SS is an aggressive malignancy that occurs predominantly in the extremities and accounts for 5–10% of soft-tissue sarcomas. PCR analysis has shown that over 90% of SS cases contain a characteristic t(11;22)(p13;q12) translocation (6), which fuses the NH₂-terminal region of SYT with the COOH-terminal region of SSX1 or SSX2 (6–8). In the chimeric fusion protein, a repression domain from SSX is replaced by a transactivation domain from SYT, and this presumably activates unknown target genes normally repressed by SSX1 or SSX2 (9).

CSS, also termed malignant melanoma of soft parts, is a rare, aggressive sarcoma of neuroectodermal origin (10) affecting muscle tendons and aponeuroses typically in the extremities (11). Over 70% of these tumors contain the t(12;22)(q13;q12) translocation, fusing the 5’ region of EWS with the 3’ region of ATF1 (12, 13). Whereas it is not precisely known how the translocation contributes to cellular transformation, a number of mechanisms have been postulated (3), including constitutive activation of ATF1 target genes, repression of growth control genes, and activation of other cAMP-responsive element binding protein (CREB)/ATF or non-CREB/ATF target genes.

DSRCT is an aggressive malignancy occurring predominantly in abdominal serosal surfaces (13). Almost 100% of these cases contain a characteristic t(11;22)(p13;q12) translocation that fuses the NH₂-terminal region of EWS with the COOH-terminal region of WT1, a tumor suppressor gene involved in a subset of Wilms’ tumors (14–18). WT1 contains three zinc fingers in the COOH-terminal region responsible for DNA binding, and it has been postulated that the loss of the proximal zinc fingers in the chimeric fusion protein converts WT1 from a transcriptional repressor to an activator (3, 19). Resulting abnormalities in WT1 target gene expression, including genes related to the early growth response family, could be causative in cellular transformation (3).

It has been shown that chimeric fusion proteins resulting from translocations can be necessary for the persistence of the tumor (20, 21), thus indelibly marking the tumor cell as a target for immunotherapy. Peptides generated from proteolytic processing of fusion regions would have a “non-self” sequence of aa, which, if displayed via MHC molecules, could result in T-cell-mediated immunity. Normal cells, on the other hand, would not contain the break-point-spanning sequence, thus distinguishing specific tumor cells and avoiding an autoimmune response. In the case of alveolar rhabdomyosarcoma, a common pediatric soft-tissue sarcoma caused by fusion of the NH₂-terminal region of PAX3 with the COOH-terminal region of FKHR (22–26), it has previously been found that a PAX3-FKHR-induced CTL line lysed murine tumor cells transfected with the full-length PAX3-FKHR cDNA (27). This demonstrates that fusion proteins can be endogenously processed in tumor cells and presented by class I MHCs on the cell surface.

In the following study, peptides derived from the sequences surrounding the breakpoints of SS, CCS, and DSRCT were assessed for the ability to bind HLA molecules and induce a primary in vitro
immune response from human T lymphocytes, which can kill human sarcoma cells.

**MATERIALS AND METHODS**

**Cell Lines.** CIR.B7 and CIR.A3 are specific transfectants of the B lymphoblastoid CIR cell line, which, in native form, expresses no endogenous HLA-A or HLA-B gene products (28), and were a gift of William Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). T2.B7 and T2.B27 are specific transfectants of the hybrid B and T lymphoblastoid T2 cell line, which is deficient in TAP1 and TAP2 gene expression (29, 30), and were a gift of Peter Cresswell (Yale University, New Haven, CT). Cell lines were maintained in complete medium (RPMI 1640 with 10% FBS, 1-glutamine, penicillin, streptomycin, sodium pyruvate, nonessential aa, and 50 μM β-mercaptoethanol).

SW982 (human SS cell line) was obtained from ATCC (Manassas, VA). By surface staining with anti-HLA-B7 antibodies (BB7.1; ATCC), it was determined to be naturally HLA-B7 positive. However, it was determined to lack the characteristic t(X;18)(p11.2;q11.2) translocation present in 90% of such tumors. Therefore, it was transfected with the fusion protein gene as described below.

The SSX-SYT full-length expression fusion cDNA was kindly provided by Dr. Colin Cooper (Haddow Laboratories, Sutton, United Kingdom). The plasmid (SYT-SSX) was precipitated with ethanol and resuspended in PBS (2 mg/ml).

The presence of the SSX-SYT fusion product was determined by reverse transcription-PCR. RNA was extracted using the Quiagen Rneasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s recommendations. One μg of total RNA was reverse transcribed using the Perkin-Elmer reverse transcription kit (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer’s recommendations using random hexamers. Single-stranded cDNA was then amplified using standard Perkin-Elmer PCR reagents for 32 cycles at 95°C for 30 s, and a final extension at 72°C for 7 min. SW982 was then loaded onto agarose gels, and the authenticity of the expected 512-bp PCR product spanning the translocation breakpoint was determined by hybridization to the SSX-SYT cDNA probe.

**Transfection of SW982 SS Tumor Cell Line.** HLA-B7-positive SW982 cells were grown to ≥80% confluence in complete medium. The cells were harvested by trypsinization and suspended in complete medium at 1 × 10^7 cells/ml. In a 0.4-cm cuvette, 0.4 ml of the cell suspension was mixed with DNA (40 μg) and electroporated in a Bio-Rad Gene Pulsar at 950 V/cm (50 μS/cm) and 20 μF (20 ms). After being allowed to stand at room temperature for 2 h, the cells were plated out in 10-cm culture dishes (37). Cells were then added to a 96-well round-bottomed plate at 2 × 10^5 cells/well. Cells were incubated for 4 h at room temperature in the presence of the appropriate peptide concentration, human β2m (2 μg/ml), and the specific anti-HLA primary antibody. This was followed by secondary antibody staining and fluorometric analysis as described. In this study, we focused on HLA-A2, -A3, -B7, and -B27 antigens because of the availability of the relevant reagents for sensitive assay of binding to these antigens, as well as the high frequency of most of these antigens in the population.

**In Vitro CTL Priming with Dendritic Cells.** Elutriated monocytes and lymphocytes were obtained from apheresis subjects from the NIH normal donor pool and screened for HLA-B7 and/or HLA-A3 expression using supertetrameric anti-HLA antibodies (ATCC). To prepare dendritic cells, monocytes from donors were cultured at 10^5 cells/ml in complete medium, 10% FBS, IL-4 (2000 units/ml), and granulocyte macrophage colony-stimulating factor (2000 units/ml). Additional identical amounts of IL-4 and granulocyte macrophage colony-stimulating factor were added every 48 h, and CD40 ligand trimmer (Immunex Corp.) was added at 1 μg/ml on day 5 (37). Cells were harvested on day 7 and stained for CD1a, CD14, CD80, CD83, CD86, and class I and class II MHC antigens.

To prime CTLs to the SS1 peptide, autologous lymphocytes were suspended in complete medium (10% FBS) and added to a 24-well plate at 4 × 10^5 cells/well. Autologous dendritic cells were irradiated with 3000 rads and mixed with the lymphocytes at 4 × 10^5 cells/well. SS1 peptide was added to each well at 10 μM. Cytokines were added to the cultures according to a previously described method (38). IL-6 (1000 units/ml) and IL-12 (10 ng/ml) were added on week 1, and IL-2 (10 units/ml) and IL-7 (5 ng/ml) were added on all subsequent weeks. Restimulations were done weekly using the same conditions. Cultures were checked every 2 weeks for relative CD4 and CD8 expression and, if necessary, depleted of CD4+ cells by passing them through an anti-CD4 column (Miltenyi Biotec Midi-MACS).

**RESULTS**

**Binding of Translocation-associated Peptides to HLA Molecules.** To determine the immunogenicity of fusion sequences associated with SS, CCS, and DSRCT, respective peptides were designed that contain all possible breakpoint-spanning 9-aa or shorter minimal epitopes and most 10-aa minimal epitopes. Peptides derived from the breakpoint region of SS were termed SS1 and SS2, the peptide derived from CCS was termed EA1, and the peptides derived from DSRCT were termed EW1 and EW2. Table 1 summarizes the peptides used and their sequences. These potentially antigenic peptides can be useful for novel immunotherapies only if they bind HLA molecules. Therefore, two distinct binding assays were used to assess specific peptide
binding to various HLA molecules. Both assays were conducted in the presence of serum, which, along with cell surface proteases, allows adequate processing of the full-length peptides to optimal lengths of 9–10 aa.

One assay uses HLA-transfected T2 cell lines (33, 34), which lack the ability to properly load cytosolic peptides onto newly synthesized class I MHC molecules in the endoplasmic reticulum. Therefore, empty and thus unstable MHC molecules only occasionally and transiently appear on the cell surface. However, an exogenously added peptide that specifically binds to the transfected MHC antigens in conjunction with β2m stabilizes the MHC antigens on the cell surface and is detectable by immunofluorescence. Using T2.B7 cells, it was found that both SS1 and SS2 peptides produce proteolytic fragments that bind HLA-B7 molecules (Figs. 1 and 2). To determine the optimal epitope for this interaction, candidate 9–10-aa peptides encoding possible HLA-B7 molecule-binding motifs were chosen from the full-length sequence (39). These peptides were chosen based on coding possible HLA-B7 molecule-binding motifs were chosen from SS2 and EA1 at least partially matching these criteria did not bind HLA-B27 molecules (data not shown). Using the same assay, all peptides were tested for the ability to stabilize HLA-A2 molecules as well, but no specificity was observed.

The second binding assay is a peptide-MHC reconstitution assay (35), in which a human cell line is briefly treated with acid, causing surface HLA antigen denaturation and dissociation of peptides and β2m. Subsequently, incubation with a specific HLA molecule-binding peptide along with β2m and the specific anti-HLA antibody of interest enables proper surface HLA antigen renaturation that can be detected by immunofluorescence. Using C1R.A3 cells, EW2 peptide (from DSRCT) stabilization of HLA-A3 molecules was observed. A 9-aa peptide (EW2-K9S) with the sequence SSYGQSEK was identified as the optimal epitope for this interaction because upon testing various candidates with HLA-A3 antigen-binding motifs (Table 3B), it bound HLA-A3 antigen with the highest affinity (39). This peptide partially matches the HLA-A3 antigen-binding motif, with a K at the COOH terminus, although it does not contain the preferred L, V, or M at position 2. In this case, the optimal epitope and full-length peptides stabilize HLA-A3 molecules with approximately superimposable titration curves (Fig. 4 and Table 3). Using the same assay, all peptides were tested for stabilization of HLA-A1 molecules, but no specificity was observed.

### Table 1. Sequences of designed peptides, along with associated translocations and sarcoma types

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Specific translocation</th>
<th>Associated sarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1</td>
<td>PQRPYGYDQ/IMPKKPA</td>
<td>STT/SSX</td>
<td>SS</td>
</tr>
<tr>
<td>SS2</td>
<td>RPYPYQDQ/IMPKKPPE</td>
<td>STT/SSX</td>
<td>SS</td>
</tr>
<tr>
<td>EA1</td>
<td>RGGBPQGGG/KIKLDDLSS</td>
<td>EWSATF1</td>
<td>CCS</td>
</tr>
<tr>
<td>EW1</td>
<td>SQSSSYYQQQ/SEKPY</td>
<td>EWS/WT1</td>
<td>DSRCT</td>
</tr>
<tr>
<td>EW2</td>
<td>SSSYGQQ/SEKPYQCDFK</td>
<td>EWS/WT1</td>
<td>DSRCT</td>
</tr>
</tbody>
</table>

![Fig. 1. FACSscan histogram showing mean cell surface fluorescence of T2.B7 cells incubated with no peptide (A, negative control, shaded), 1.0 μM SS2 (B), 1.0 μM SS1 (C), and 1.0 μM SS1-M10Q (D, minimal epitope)]. The experiment was repeated three times with comparable results.

![Fig. 2. Binding of SS-derived peptides to HLA-B7 molecules. T2.B7 cells, which do not stably express surface class I MHCs, were incubated overnight in the presence of SS1, SS2, or SS1-M10Q peptide along with human β2m and stained the following day for cell surface HLA-B7 expression. FI reflects the fractional increase in cell surface fluorescence in the presence of a given peptide concentration as compared with a negative control (no peptide added). The experiment was repeated three times with comparable results.](image)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Associated sarcoma</th>
<th>Specific HLA interaction</th>
<th>P_{K_a} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1</td>
<td>SS</td>
<td>B7</td>
<td>0.1</td>
</tr>
<tr>
<td>SS2</td>
<td>SS</td>
<td>B7</td>
<td>0.3</td>
</tr>
<tr>
<td>EA1</td>
<td>CCS</td>
<td>B27</td>
<td>8.1</td>
</tr>
<tr>
<td>SS2</td>
<td>SS</td>
<td>B27</td>
<td>4.0</td>
</tr>
<tr>
<td>EW2</td>
<td>DSRCT</td>
<td>A3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

![Table 2. Specific HLA antigen interactions for each translocation-associated peptide, along with approximate relative binding avidities](image)
In A, SS1-M10Q is the selected epitope for HLA-B7 antigen binding, and in B, EW2-K9S is the selected epitope for HLA-A3 antigen binding. Full-length peptides are shown at the top of each table.

**Table 3** Optimal epitope candidate peptides for HLA-B7-(A) or HLA-A3-(B) antigen binding, along with sequences and approximate FI_{1.5} values.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>FI_{1.5} for HLA-B7 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1</td>
<td>PQQPGYDQIMPKPAF</td>
<td>0.1</td>
</tr>
<tr>
<td>SS2</td>
<td>RFPGYDQIMPKPAE</td>
<td>0.3</td>
</tr>
<tr>
<td>SS1-M10Q</td>
<td>QRPGYDQIMPKPAE</td>
<td>0.02</td>
</tr>
<tr>
<td>SS1-P10R</td>
<td>RPQPGYDQIMPK</td>
<td>2.0</td>
</tr>
<tr>
<td>SS1-I9</td>
<td>QRPGYDQIMQ</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SS2-E10D</td>
<td>DQIMPKPAE</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SS2-K9G</td>
<td>GYDQIMPK</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SS2-K9Y</td>
<td>GYDQIMPK</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SS2-M9</td>
<td>RPQPGYDQIMQ</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>FI_{1.5} for HLA-A3 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EW2</td>
<td>SSSYQQQSKGYPYCDPK</td>
<td>0.1</td>
</tr>
<tr>
<td>EW2-K9S</td>
<td>SSSYQQQSKGYPYCDPK</td>
<td>0.1</td>
</tr>
<tr>
<td>EW2-K10S</td>
<td>SSSYQQQSKGYPYCDPK</td>
<td>0.1</td>
</tr>
<tr>
<td>EW2-Y9</td>
<td>SSYQQQSKGYPYCDPK</td>
<td>&gt;100</td>
</tr>
<tr>
<td>EW2-K9S</td>
<td>SSSYQQQSKGYPYCDPK</td>
<td>&gt;100</td>
</tr>
<tr>
<td>EW2-Y10S</td>
<td>SSSYQQQSKGYPYCDPK</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

**Determination of MHC-interacting Residues.** To determine which aa in the SS1-M10Q minimal epitope are primarily responsible for interaction with HLA-B7 molecules, a series of single mutation 10-aa peptides were made in which Ala was substituted for each position (Table 4). HLA-B7 molecule binding of the mutated peptides was then compared with that of the wild-type SS1-M10Q peptide using FI_{1.5} values. These values reflect the peptide concentration required for a 150% increase in T2.B7 cell surface fluorescence as compared with a negative control (no peptide added). Whereas FI_{1.5} (indicating a 50% fluorescence increase) has previously been used to compare binding affinities, this series of peptides gave high background FI values, and affinities were better distinguished at greater FI values (Fig. 5). Ala substitution at positions 2, 3, 6, and 10 drastically reduced HLA-B7 molecule binding, with FI_{1.5} values greater than 2.5 orders of magnitude above that of SS1-M10Q (Fig. 5 and Table 4). Of the remaining residues, a mutation at position 1 resulted in binding similar to that seen with SS1-M10Q, reflecting either a minor role or no role in MHC antigen binding. Furthermore, mutations at positions 4–5 and 7–9 resulted in FI_{1.5} increases of 1–2 orders of magnitude, reflecting intermediate but not primary importance in MHC antigen binding.

**Generation of CTLs Specific for SS-derived Peptide.** To test the immunological relevance of the peptide-MHC interaction, euthanized monocytes and lymphocytes (>95% purity) from normal donors were then stained with anti-HLA-B7 (monoclonal antibody BB7.1; ATCC) and flow microfluorometry by FACSscan, and cells from positive donors were used to induce a SS1-specific CTL response stimulated by autologous monocyte-derived dendritic cells matured with CD40 ligand. These dendritic cells expressed high levels of CD80, CD83, CD86, and class I and class II MHC antigens, low levels of CD14, and no CD1a, consistent with a mature phenotype (data not shown). In one of four donors, a SS1-specific class I-restricted CD8+ CTL response was evident after four in vitro restimulations with SS1-pulsed autologous dendritic cells. As seen in Fig. 6A, the CTLs specifically lysed T2.B7 targets pulsed with as low as 1 nm SS1-M10Q. This lysis is specific for the peptide in combination with HLA-B7 antigen and not for a different T2-specific determinant because untransfected T2 cells (those expressing HLA-A2 and -B5) were not lysed by the CTLs (data not shown). Furthermore, CIR.B7 targets, which express HLA-B7 even in the absence of peptide, were also specifically lysed when pulsed with SS1-M10Q, ruling out the possibility that the CTLs are specific for a determinant on HLA-B7 antigen alone (Fig. 6B).

After the establishment of the SS1-specific CTL line, the relevant donor’s peripheral blood mononuclear cells were HLA-typed by DNA typing, using sequence-specific primer PCR (40) with confirmation by sequencing to check for HLA-B7 subtype. Surprisingly, it was found that the donor is HLA-B13 and is not in fact HLA-B7, indicating that the BB7.1 monoclonal anti-HLA-B7 initially used for HLA typing most likely cross-reacts with at least one of the HLA-B alleles present. This cross-reactivity, which probably occurred with HLA-B42 because of known sequence homology, appears to extend to T-cell receptor recognition, given the ability of T cells from this donor to recognize antigen presented by HLA-B7 molecules and the lack of killing on HLA-B7 targets (CIR.B7) not expressing the specific peptide (Fig. 6B). As further confirmation of the HLA-B7 restriction of killing, the lysis was inhibited by the addition of HLA-B7restricted CD8+ CTL response was evident after four in vitro restimulations with SS1-pulsed autologous dendritic cells. As seen in Fig. 6A, the CTLs specifically lysed T2.B7 targets pulsed with as low as 1 nm SS1-M10Q. This lysis is specific for the peptide in combination with HLA-B7 antigen and not for a different T2-specific determinant because untransfected T2 cells (those expressing HLA-A2 and -B5) were not lysed by the CTLs (data not shown). Furthermore, CIR.B7 targets, which express HLA-B7 even in the absence of peptide, were also specifically lysed when pulsed with SS1-M10Q, ruling out the possibility that the CTLs are specific for a determinant on HLA-B7 antigen alone (Fig. 6B).

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**Fig. 3.** Binding of SS- and CCS-derived peptides to HLA-B27 antigen. T2.B27 cells, which do not stably express class I MHCs, were incubated overnight in the presence of SS2 (from SS) or EA1 (from CCS) peptide along with human β₂m and stained the following day for cell surface HLA-B27 expression. FI reflects the fractional increase in cell surface fluorescence in the presence of a given peptide concentration as compared with a negative control (no peptide added). The experiment was repeated three times with comparable results.

**Fig. 4.** Binding of DSRCT-associated peptides to HLA-A3 molecules. CIR.A3 cells were treated with acid, thereby denaturing surface HLA-A3 molecules, and then incubated with EW2 or EW2-K9S peptide, human β₂m, and anti-HLA-A3 for 4 h. The cells were then stained for cell surface HLA-A3 expression. FI reflects the fractional increase in cell surface fluorescence in the presence of a given peptide concentration as compared with a negative control (no peptide added). The experiment was repeated two times with very similar results (the FI_{1.5} was consistently around 0.1 μM for both EW2 and EW2-K9S binding to HLA-A3 molecules).
antibody (the same one that reacts with the donor’s cells) in the
lytic assay (Fig. 7).

Lysis of Human SS Tumor Cells Expressing HLA-B7 and the SYT-SSX Fusion Protein. To determine whether the SS1-M10Q epitope can be processed and presented from endogenously ex-
pressed full-length SYT-SSX fusion protein in tumor cells and
presented by HLA-B7 molecules on the surface of the tumor cells
for lysis by CTLs, we tested the ability of the epitope-specific CTL
to kill HLA-B7+ human SS tumor cells expressing the full-

length SYT-SSX fusion protein. The human SS tumor line SW982
(obtained from ATCC) was found to express HLA-B7 by surface
staining (data not shown) but surprisingly was one of the minority
of such tumors that do not express the fusion protein. Therefore,
we transfected the full-length SYT-SSX fusion protein gene into
this cell line, as described in “Materials and Methods.” Clones
expressing SYT-SSX mRNA were identified by reverse transcrip-
tion-PCR. These cells were then tested as targets for lysis by the
SS1-M10Q-specific human CTL line. We took advantage of the
existence of the untransfected SW982 tumor cell line, which ex-
presses the same level of HLA-B7 but lacks the fusion protein, to
use it as a specificity control. Two independent experiments
showed that specific lysis of the SYT-SSX-expressing human
SW982 tumor cells was significantly higher than the background
lysis of the control untransfected SW982 tumor line (Fig. 8). Thus,
the SYT-SSX fusion protein is endogenously processed and pre-
sent in human SS cells for expression of the SS1-M10Q epitope
in association with HLA-B7 molecules on the surface of the tumor
cells and for lysis by epitope-specific human CTLs. This result
confirms that this fusion protein is a bona fide tumor antigen that
is unique to the tumor because the epitope spans the translocation
breakpoint, which is not present in any normal cells, and is
therefore a potential target for immunotherapy.

Table 4 Comparison of approximate relative HLA-B7 antigen binding avidities for
SS1-M10Q and respective Ala-derivatives, determined by a T2 cell binding assay
(see “Materials and Methods”)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>FI_{1.5} for HLA-B7 (µM)</th>
</tr>
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<tbody>
<tr>
<td>SS1-M10Q</td>
<td>QRPYGYDQIM</td>
<td>0.03</td>
</tr>
<tr>
<td>SS1-M10Q-1A</td>
<td>ARPYGYDQIM</td>
<td>0.1</td>
</tr>
<tr>
<td>SS1-M10Q-2A</td>
<td>QAPYGYDQIM</td>
<td>10</td>
</tr>
<tr>
<td>SS1-M10Q-3A</td>
<td>QRAYGYDQIM</td>
<td>&gt;10</td>
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<td>SS1-M10Q-4A</td>
<td>QRPAYDQIM</td>
<td>0.3</td>
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<td>SS1-M10Q-5A</td>
<td>QRPYADQIM</td>
<td>1.0</td>
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<tr>
<td>SS1-M10Q-6A</td>
<td>QRPYADQIM</td>
<td>&gt;10</td>
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<td>SS1-M10Q-7A</td>
<td>QRPYGADQIM</td>
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<td>SS1-M10Q-8A</td>
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<td>SS1-M10Q-9A</td>
<td>QRPYDGQAM</td>
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<td>SS1-M10Q-10A</td>
<td>QRPYGDAQIA</td>
<td>10</td>
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Fig. 5. Comparison of HLA-B7 antigen binding between SS1-M10Q and associated
Ala-substituted peptides. T2.B7 cells, which do not stably express surface class I MHCs,
were incubated overnight in the presence of SS1-M10Q or an indicated Ala-substituted
peptide along with human /H9252
2 m and stained the following day for cell surface HLA-B7
molecule expression. FI reflects the fractional increase in cell surface fluorescence in the
presence of a given peptide concentration as compared with a negative control (no peptide
added). The experiment was done twice with comparable results. A, FI values for each
peptide at all concentrations are shown. B, for clarity at lower concentrations, only FI
values less than 5.0 are shown.

Fig. 6. A, lysis of SS1-M10Q-pulsed T2.B7 targets by SS1-specific CTLs. A standard
^{51}Cr release assay (see “Materials and Methods”) was used to measure lysis (indicated by the
percentage of release) of peptide-pulsed target cells. Titrating peptide concentrations
were used to pulse the targets, and background lysis (no peptide added to targets) is
indicated by the horizontal line at 11.5%. An E:T ratio of 50:1 was used. The experiment
was repeated three times with comparable results. B, corresponding lysis of SS1-M10Q-
pulsed C1R.B7 targets. Both the E:T ratio and peptide concentrations were titered. The
experiment was repeated two times with comparable results.

SARCOMA TRANSLOCATION FUSION PROTEIN TUMOR ANTIGENS

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molecule interactions. HLA antigen-binding motifs alone do not guarantee peptide-HLA matching; particular HLA antigen-binding motifs are sometimes still needed for some sequences to bind. These results indicate that peptides not matching particular HLA antigen-binding motifs sequences can bind. Furthermore, HLA-B27 antigen-binding motif sequences in EA1 high-affinity binding in the face of this nonconservative alteration. PR at positions 2 and 3 are reversed in SS1-M10Q, which has R at position 2 and P at position 3. It is unexpected to observe such binding in this particular case may be as high as can easily be achieved. It is interesting to note that in the case of SS1-M10Q (from SS) binding to HLA-B7 molecules, the classical binding motif (39) is not fulfilled. Although M can frequently substitute for L as the COOH-terminal residue in class I MHC-binding motifs, the anchor positions PR at positions 2 and 3 are reversed in SS1-M10Q, which has R at position 2 and P at position 3. It is unexpected to observe such high-affinity binding in the face of this nonconservative alteration. Furthermore, HLA-B27 antigen-binding motif sequences in EA1 (from CCS) and SS2 (from SS) failed to bind HLA-B27 molecules, although the activity of the full-length peptides shows the presence of sequences that can bind. These results indicate that peptides not matching particular HLA antigen-binding motifs are sometimes still capable of binding the corresponding HLA molecules and that good HLA antigen-binding motifs alone do not guarantee peptide-HLA molecule interactions.

It was similarly shown by Yotnda et al. (41) that an acute lymphoblastic leukemia breakpoint-associated peptide binds to HLA-A2.1 antigens. This peptide was shown to induce a specific CTL response in peripheral blood lymphocytes from normal HLA-A2+ donors, and specific CTLs were also found to be present in the bone marrow of a HLA-A2+ acute lymphoblastic leukemia patient. It was also shown by Ohminami et al. (42) that a specific CTL response can be raised to an HLA-A24-binding peptide within the WT1 gene product, which is overexpressed in many leukemias. This peptide, however, is not associated with a fusion breakpoint. In addition, Gambacorti-Passerini et al. (43) predicted and tested a number of HLA antigen-binding motifs among fusion proteins, including the EWS/FLI1 product from Ewing’s sarcoma and the EWS/ATF1 product from CCS. Two distinct Ewing’s sarcoma-derived peptides bound specifically to HLA-Cw0702 molecules, although a primary in vitro cellular immune response could not be obtained from HLA-Cw0702 donor peripheral blood mononuclear cell samples stimulated with the same peptides (44). These results suggest that HLA binding is not always sufficient to generate a cellular immune response. Because Ala-substituted peptides were not examined, it is also possible that the lack of a T-cell response was due to low-affinity peptide-MHC interactions.

In the present study, it was shown that several sarcoma-associated peptides bind common class I HLA molecules. In the case of SS1 peptide binding to HLA-B7 molecules, a specific CTL response was generated from lymphocytes initially typed to be HLA-B7+ by binding of the BB7.1 anti-HLA-B7 monoclonal antibody. Subsequently, this particular donor was discovered instead by DNA typing to be HLA-B42+ (and HLA-B13+), raising the question of cross-presentation of the SS1-M10Q peptide by the HLA-B7-related HLA-B42 molecule to CTLs, which are perhaps specific for the peptide bound to either HLA-B7 or HLA-B42 molecules. Consistent with this interpretation, Chelvanayagam (45) and Zhang et al. (46) have shown that HLA-B7 and HLA-B42 molecules share conserved peptide-binding pockets at positions P2 and P1 (the COOH-terminal residue). In particular, the polymorphic positions in each pocket, which are primarily responsible for creating a unique peptide-binding environment, are the same for each, including Y9, Y67, and I66 in P2 and Y116 in P0. It has also been shown by Rammensee et al. (39) that the second and COOH-terminal peptide positions within the HLA-B7 molecule binding motif are critical, and this matches the binding pockets that are conserved between HLA-B7 and HLA-B42.

This study provides the first evidence that the SYT-SSX fusion protein encodes a tumor neoantigen capable of stimulating human
sarcomas. These results suggest a potential role for translation-specific peptides in novel immunotherapies for these sarcomas.

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