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Proteasome-Assisted Identification of a SSX-2-Derived Epitope Recognized by Tumor-Reactive CTL Infiltrating Metastatic Melanoma[1]

Maha Ayyoub,[2]* Stefan Stevanovic,[†] Ugur Sahin,[3‡] Philippe Guillaume,[§] Catherine Servis,[¶] Donata Rimoldi,[§] Danila Valmori,* Pedro Romero,* Jean-Charles Cerottini,[§] Hans-Georg Rammensee,[†] Michael Pfreundschuh,[‡] Daniel Speiser,[4]* and Frédéric Lévy[4§]

The tumor Ag SSX-2 (HOM-MEL-40) was found by serological identification of Ags by recombinant expression cloning and was shown to be a cancer/testis Ag expressed in a wide variety of tumors. It may therefore represent a source of CD8$^+$ T cell epitopes useful for specific immunotherapy of cancer. To identify potential SSX-2-derived epitopes that can be recognized by CD8$^+$ T cells, we used an approach that combined: 1) the in vitro proteasomal digestion of precursor peptides overlapping the complete SSX-2 sequence; 2) the prediction of SSX-2-derived peptides with an appropriate HLA-A2 binding score; and 3) the analysis of a tumor-infiltrated lymph node cell population from an HLA-A2$^+$ melanoma patient with detectable anti-SSX-2 serum Abs. This strategy allowed us to identify peptide SSX-2$_{41-49}$ as an HLA-A2-restricted epitope. SSX2$_{41-49}$-specific CD8$^+$ T cells were readily detectable in the tumor-infiltrated lymph node population by multimer staining, and CTL clones isolated by multimer-guided cell sorting were able to lyse HLA-A2$^+$ tumor cells expressing SSX-2. *The Journal of Immunology,* 2002, 168: 1717–1722.

The identification of tumor Ags able to elicit an immune response in cancer patients has opened the way for specific immunotherapy of cancer (1, 2). Two major categories of tumor Ags are currently used for experimental antitumor immunotherapy: 1) cancer-testis (CT)[5] Ags expressed in tumors from different histological origins but not in normal tissues, testis excepted; and 2) melanocyte lineage differentiation Ags expressed in both melanomas and normal melanocytes but not in other normal tissues (1, 2). Whereas differentiation Ags are useful for immunotherapy of melanoma, CT Ags can be used for the treatment of a wide range of tumor types.

Serological identification of Ags by recombinant expression cloning (SEREX), a method based on the cloning of genes encoding proteins recognized by high titer IgG Abs from cancer patients' sera, has led to the identification of a series of Ags including the SSX, NY-ESO, and SCP CT Ag families (3–8). Spontaneous humoral immune responses in cancer patients indicate that SEREX-defined proteins are immunogenic and may thus represent interesting targets for immunotherapy of cancer. Because CTLs represent a major arm of the immune response against cancer, the elicitation of a specific CTL response against tumor Ags is one of the main aims of current immunotherapy trials. Therefore, it is crucial to define CD8$^+$ T cell epitopes in SEREX-defined Ags. However, despite the large number of SEREX-defined Ags, no CD8$^+$ T cell epitopes have been characterized thus far in SEREX proteins other than NY-ESO-1 (9, 10).

HOM-MEL-40 was cloned in the initial SEREX study using serum from a metastatic melanoma patient (6). The sequence of the *HOM-MEL-40* gene was identical with that of *SSX-2*, identified as one of the two genes involved in the translocation t(X;18)(p11.2; q11.2) found in 70% of synovial sarcomas (7, 11). Expression analysis performed by Northern blot and RT-PCR demonstrated the presence of SSX-2 transcripts in a significant proportion of human cancers, as for instance in melanomas (35%), head and neck cancers (35%), lymphomas (36%), and colon carcinomas (12%), but not in normal tissues with the exception of testis (7, 12). These findings allowed the classification of SSX-2 as a CT Ag. In addition, Abs against SSX-2 were found in 10% of patients with melanoma (7). Altogether these data highlighted SSX-2 as a potentially interesting target for immunotherapy of a large number of malignancies. However, the cellular immune response against SSX-2 in cancer patients has not been documented and no SSX-2-derived T cell epitopes have been identified.

A simple approach used to identify CD8$^+$ T cell epitopes in a given protein is based on the prediction of peptides displaying the appropriate MHC class I binding motif and on the subsequent in vitro generation of CTLs against the predicted peptides (13). This approach, named reverse immunology, has been applied to a number of tumor-associated proteins but has led, in several instances, to the generation of peptide-specific CTLs that are unable to lyse Ag-presenting tumor cells (14, 15). This was shown to be mainly

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[5] Abbreviations used in this paper: CT, cancer-testis; SEREX, serological identification of Ags by recombinant expression cloning; TILN, tumor-infiltrated lymph node; TFA, trifluoroacetic acid; hr, human recombinant.

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due to the fact that these peptides are normally not efficiently processed by the cellular machinery (16, 17). Recently, an improved reverse immunology strategy has been described (18). In that study, in vitro digestions of synthetic oligopeptides with purified proteasome led to the identification of cleavage products the C termini of which were compatible with the peptide-binding motifs for particular HLA class I molecules. The likelihood that the candidate HLA class I ligands were indeed processed in vivo was high, because it is now accepted that proteasomes generally generate the exact C termini of MHC class I-restricted antigenic peptides (19). Synthetic nonapeptides displaying the identified C termini were then used to stimulate PBMC and were shown to elicit a CTL response (18). Validation of this approach was provided by the demonstration that the derived CTL were able to lyse target cells expressing both the studied protein and the appropriate MHC restriction element. Although this strategy was a clear improvement over the original motif-based peptide prediction, the authors did not determine whether a natural immune T cell response against any of the identified peptide tumor Ags took place in vivo.

Here we associated the in vitro proteasomal degradation assay of overlapping SSX-2 peptides with the prediction of HLA-A2-binding peptides based on anchor residues to identify a natural T cell response in a tumor-infiltrated lymph node (TILN) of a melanoma patient seropositive for SSX-2 and whose tumor expressed SSX-2. We report the identification of an HLA-A2-restricted peptide derived from SSX-2 against which a natural $CD8^+$ T cell response was detectable.

Materials and Methods

Epitope prediction and synthetic peptides

Epitope prediction was performed as described (20). Briefly, potential HLA-A*0201 ligands from the sequence of SSX-2 were selected using a matrix pattern suitable for the calculation of nonamer or decamer peptides fitting to the HLA-A*0201 motif. Such motif predictions are available on our web page at http://www.syfpeithi.de.

Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the *N*-(9-fluorenyl)methoxycarbonyl-/*tert*-butanol strategy. After removal from the resin by treatment with trifluoroacetic acid (TFA)-phenol-ethanedithiol-thioanisole-water (90:3.75:1.25:2.5:2.5 by volume) for 1 or 3 h (arginine-containing peptides), peptides were precipitated from methyl-*tert*-butyl ether, washed once with methyl-*tert*-butyl ether and twice with diethyl ether, and resuspended in water before lyophilization. Synthesis products were analyzed by HPLC (Varian star; Zinsser, Munich, Germany) and MALDI-TOF mass spectrometry (G2025A; Hewlett-Packard, Waldbronn, Germany). Peptides of <80% purity were purified by preparative HPLC.

Cell lines and clones

Surgically resected metastatic LN from patient LAU 50 was finely minced with needles in sterile RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS. Cell suspensions were placed in 24-well tissue culture plates (Costar, Cambridge, MA) in 2 of IMDM (Life Technologies, Basel, Switzerland) supplemented with 0.24 mM aspartic acid, 0.55 mM arginine, 1.5 mM glutamine, 8% pooled human serum (CTL medium), 100 U/ml human recombinant (hr) IL-2 (Glaxo, Geneva, Switzerland; kindly provided by Dr. M. Nabholz, Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland), and 10 ng/ml hrIL-7 (Biosource International, Camarillo, CA). Cells were cultured 2–3 wk before FACS or ELISPOT. SSX-2-specific CTL clones were derived from TILN by limiting dilution cultures in the presence of irradiated allogeneic PBMC, PHA, and hrIL-2 as described elsewhere after isolation of specific cells by multimer guided cell sorting. Clones were derived from wells with a probability of clonality higher than 90% according to single-hit Poisson distribution. Melanoma cell lines Me 275 and Me 260 were established at the Ludwig Institute for Cancer Research, Lausanne Branch, from surgically excised melanoma lesions from patients LAU 50 and LAU 149, respectively. SK-MEL-37 and SK-MEL-23 cells were kindly provided by Dr. Y. T. Chen (Ludwig Institute for Cancer Research, New York Branch, New York, NY). Tumor cell lines (Me 290, Me 260, SK-MEL-23, and SK-MEL-37) and COS-7 cells were maintained in DMEM and the $HLA\text{-}A2^+$ human mutant cell line CEMx721.T2 (T2 thereafter) in RPMI 1640 supplemented with 10% FCS.

Proteasome purification and peptide digestion

Proteasomes were purified from outdated human blood as previously described (17). Synthetic peptide (2 nmol) was digested with 16 μg purified proteasome for 10, 20, 40, and 80 min at 37°C. The reactions were stopped by adding 2% TFA. After lyophilization, the samples were analyzed by MALDI-TOF as described (17). The identification of proteolytic fragments was based on digestion times that were variable for each peptide. However, the time point was selected so that the mixture still contained relative excess of precursor peptides, thereby avoiding overdigestion of the precursors. Each peptide precursor was digested at least twice, using two different batches of purified proteasome, and yielded similar proteolytic products, confirming that the results obtained by this assay are reproducible.

PCR analysis

RNA extraction from cell lines was performed with Trizol reagent (Life Technologies, Basel, Switzerland). cDNA synthesis was performed as previously described (21), and aliquots (equivalent to 100 ng of RNA) were used for different PCR using a Qiagen HotStar *Taq* polymerase Master Kit (Basel, Switzerland). SSX-2 was amplified as described (22). Actin was amplified on each cDNA sample to assess the quality and quantity of input RNA.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assay was performed in nitrocellulose-lined 96-wells microplates (MAHA S45; Millipore, Bedford, MA) using a IFN-γ ELISPOT kit (DIACLONE, Besançon, France) according to the manufacturer's instructions with minor modifications. Plates were coated overnight with human IFN-γ-specific Ab and washed six times. T2 cells (5×10^4/well) were then added together with the indicated number of responder T cells and peptide (1 μM where indicated). After incubation for 20 h at 37°C, cells were removed and plates were developed with a second (biotinylated) human IFN-γ-specific Ab and streptavidin-alkaline phosphatase. Spots were counted using BIOREADER 2000 (BIO SYS, Frankfurt, Germany).

Tetramer staining and flow cytometry immunofluorescence analysis

A2/SSX-2_{41-49} multimeric complexes were synthesized as described (23). Cells were stained with PE-labeled multimers (0.25 μg/10^5 cells) in 20 μl PBS, 5% FCS during 20 min at 25°C, then 20 μl of a 1/25 dilution of anti-$CD8^{FITC}$ mAb (BD Biosciences, San Jose, CA) were added and incubated for an additional 30 min at 4°C. Cells were washed once in the same buffer, analyzed, and/or sorted by flow cytometry (FACScan or FACSVantage SE; BD Biosciences). Data analysis was performed using Cell Quest software.

Chromium release assay

Ag recognition was assessed using target cells labeled with ^{51}Cr for 1 h at 37°C and washed three times. Labeled target cells (1000 cells in 50 μl) were then added to effector cells (100 μl) at the indicated E:T ratios in V-bottom microwells in the presence or absence of antigenic peptide (50 μl) at the indicated concentrations. Chromium release was measured in supernatant (100 μl) harvested after 4 h incubation at 37°C. The percent specific lysis was calculated as: 100 × [(experimental − spontaneous release)/(total − spontaneous release)].

Transient transfection and IFN-γ release assay

SSX-2 and SSX-4 encoding cDNA was cloned in pcDNA3.1 plasmid (Invitrogen; Life Technologies, Basel, Switzerland). COS-7 cells were transiently transfected with SSX-2- or SSX-4-encoding plasmids and with a plasmid encoding HLA-A2 using FuGENE reagent according to the manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland). Transfected cells were then tested for their ability to stimulate the release of IFN-γ by CTL clones. In brief, CTL (25,000/well) were added to transfected cells (10,000/well) in 96-well flat-bottom plates in 200 μl/well IMDM supplemented with 10% human serum and 20 U/ml hrIL2. After a 24-h incubation at 37°C, supernatants were collected, and the IFN-γ content was determined by ELISA (BioSource Europe, Fleurus, Belgium).

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Results

Mapping the proteasomal cleavage sites in SSX-2

We tested whether the in vitro proteasomal digestion of the 188-aa tumor Ag SSX-2 could be used to restrict the number of candidate epitopes among peptides predicted to bind to HLA-A2 molecules. To this end, a set of 14 peptides 22 aa long and 1 with 20 aa, which covered the complete SSX-2 protein sequence (Fig. 1), was synthesized so that each peptide overlapped with the preceding one by 10 aa. These peptides were incubated with standard proteasome purified from human erythrocytes, and the digested products were subsequently analyzed by mass spectrometry. A summary of the identified cleavage sites is shown in Fig. 1. The number of SSX-2 nonamer and decamer peptides predicted to bind to HLA-A2 molecules (i.e., with a score of >15) was 29 (data not shown). This binding prediction is mainly based on the identity of the anchor residues and has been described elsewhere (http://www.syfpeithi.de; Ref. 20). Of 17 HLA-A2-restricted high or intermediate binding affinity nonamers and 12 decamers predicted in the SSX-2 protein, the C terminus of only 8 nonamers and 4 decamers was generated by purified proteasome (Table I). Taken together, these results indicate that <50% of the predicted high or intermediate binding affinity HLA-A2-restricted nonamers and <35% of the predicted decamers are likely to be generated intracellularly by proteasomal degradation.

Screening of reactivity to predicted SSX-2-derived peptides revealed SSX-2$_{41-49}$-specific T cells in a short term cultured melanoma-infiltrated lymph node

The accumulation of T lymphocytes specific for a given tumor Ag in melanoma lesions is one of the main features of a natural immune response against this Ag (24). HLA-A*0201^{+} patient LAU 50 was operated on for a primary malignant melanoma in 1992, s.c. metastases in 1993, and lymph node metastases in 1995 and has remained tumor free since then. While screening a series of patients' sera for the presence of anti-SSX-2 Abs, a sample obtained in 1999 from LAU 50 was found positive (data not shown). RT-PCR of RNA from LAU 50 metastatic lesion excised in 1993 showed that the tumor expressed SSX-2 (data not shown). SSX-2 was also expressed in the tumor cell line Me 275 derived from the TILN excised in 1995 (Fig. 4*A*). As previously reported (10, 25, 26), CTL clones specific for several tumor Ags (NY-ESO-1$_{157-165}$, Camel$_{1-11}$, and MAGE-A10$_{254-262}$) have been derived from this TILN. On the basis of these data, we performed an ELISPOT to assess the recognition of the above predicted SSX-2-derived peptides (Table I) by the short term-cultured TILN population obtained from LAU 50 in 1995. As shown in Table I, peptides SSX-2$_{41-49}$ (KASEKIFYV) and SSX-2$_{40-49}$ (MKASEKIFYV) induced the production of IFN-γ by cells from the TILN (69 and 39 spots, respectively). The other 11 predicted peptides gave only 3 to 12 spots per well, comparable with the background given by the control HIV-derived peptide. Because the nonamer peptide SSX-2$_{41-49}$ has a higher HLA-A2-binding score than the decamer peptide SSX-2$_{40-49}$ (Table I), we synthesized PE-labeled HLA-A2 multimers containing peptide SSX-2$_{41-49}$ and used them to stain the TILN population. As shown in Fig. 2*A*, 0.64% of CD8^{+} cells in the TILN population stained clearly positive. Altogether these data demonstrate the presence of SSX-2$_{41-49}$-specific CD8^{+} T cells within the TILN obtained from LAU 50.

Table I. *Recognition of SSX-2-derived peptides by LAU 50 TILN*[a]

Peptide	Sequence	Score	No. of Spots
SSX-2$_{5-13}$	DAFARRPTV	17	5
SSX-2$_{7-15}$	FARRPTVGA	15	5
SSX-2$_{15-24}$	AQIPEKIQKA	16	3
SSX-2$_{16-24}$	QIPEKIQKA	21	4
SSX-2$_{40-49}$	MKASEKIFYV	16	39
SSX-2$_{41-49}$	KASEKIFYV	22	69
SSX-2$_{50-59}$	YMKRKYEAMT	15	3
SSX-2$_{53-61}$	RKYEAMTKL	15	4
SSX-2$_{57-65}$	AMTKLGFKA	16	4
SSX-2$_{58-67}$	MTKLGFKATL	17	7
SSX-2$_{59-67}$	TKLGFKATL	19	12
SSX-2$_{103-111}$	RLQGISPKI	23	6

[a] HLA-A2-binding peptides (as predicted by motif-based algorithm) for which the C terminus could be generated in vitro by proteasome digestion of 22-aa precursors (Fig. 1). Theoretical binding score given by motif-based prediction algorithm in SYFPEITHI database (http://www.syfpeithi.de; Ref. 20). Number of spots obtained by IFN-γ ELISPOT of LAU 50 TILN. Short term-cultured TILN from patient LAU 50 (2×10^4 cells/well) were tested by IFN-γ ELISPOT in the presence of T2 cells (5×10^4 cells/well) alone or in the presence of the indicated peptide in duplicates. The number of spots obtained in the absence of peptide or in the presence of control peptide HIV-RT$_{476-484}$ was 3 and 13, respectively.

Functional analysis of SSX-2$_{41-49}$-specific CD8^{+} T cell clones isolated from TILN shows SSX-2- and HLA-A2-dependent tumor recognition

SSX-2$_{41-49}$ specific clones were obtained by limiting dilution cloning of multimer^{+}CD8^{+} T cells isolated by multimer guided flow cytometry cell sorting from the TILN of patient LAU 50. Clone LAU 50 E2.4 was specifically stained by A2/SSX-2$_{41-49}$

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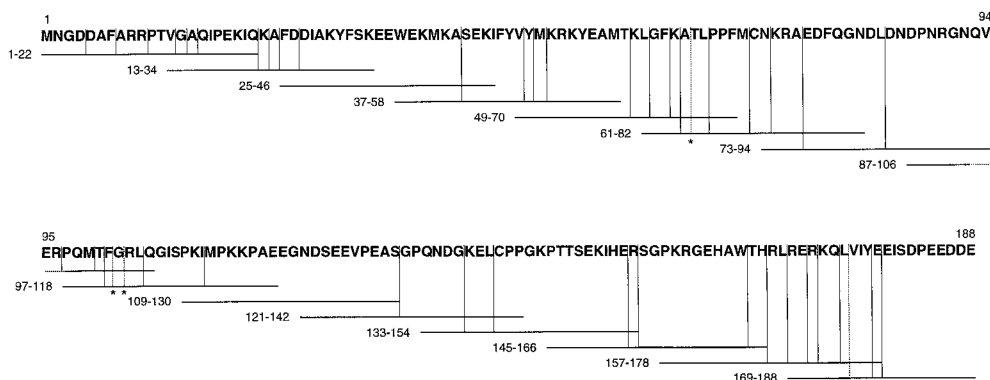

FIGURE 1. Cleavage of SSX-2 oligopeptides by proteasome. A set of 15 overlapping peptides covering the entire SSX-2 protein sequence (in one-letter code) was digested by human proteasome and analyzed by mass spectrometry. The numbers of each peptide correspond to the position of the first and last amino acid of the peptide within the protein. A cleavage is indicated by a solid vertical bar, which intersects with the peptide in which it was detected. Bars with dotted line and asterisks indicate that the same cleavage site was detected in two overlapping peptides.

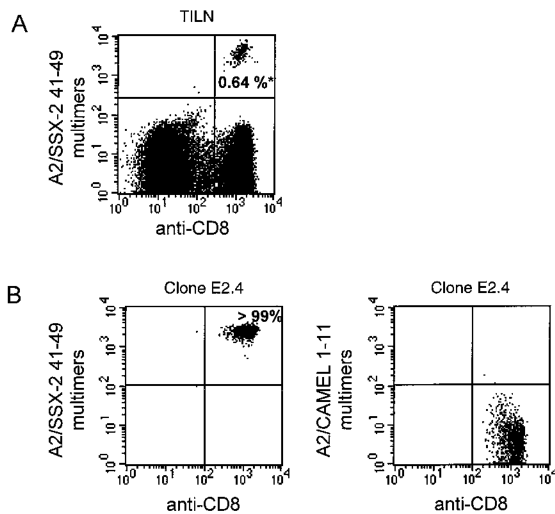

FIGURE 2. Enumeration of SSX-2_{41-49}-specific cells in LAU 50 TILN and isolation of specific clonal populations. *A,* Short term-cultured TILN from patient LAU 50 were stained with PE-labeled A2/SSX-2_{41-49} multimers and anti-$CD8^{FITC}$ mAb and analyzed by flow cytometry. The number in the upper right quadrant represents the percentage of $multimer^+$ cells within $CD8^+$ T lymphocytes. *B,* Clone LAU 50 E2.4 was stained with PE-labeled A2/SSX-2_{41-49} (*left*) or A2/$CAMEL_{1-11}$ (*right*) multimers and anti-$CD8^{FITC}$ mAb and analyzed by flow cytometry.

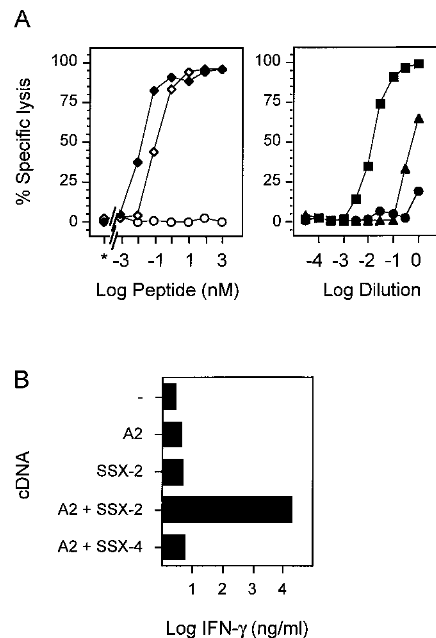

FIGURE 3. Functional characterization of SSX-2_{41-49} specific CTL clone LAU 50 E2.4. *A,* Lytic activity of the clone was tested in a ^{51}Cr release assay against T2 cells at an E:T ratio of 10:1 in the absence (*) or in the presence of exogenously added peptides at the indicated concentrations. *Left,* Peptide SSX-2_{41-49} (◆), SSX-2_{40-49} (◇) or $MelanA_{26-35\ A27L}$ (○) were tested. Lysis of T2 cells pulsed with proteasome-digested peptide products was also tested (*right*). Three-fold dilutions, starting from 1 μM, of the precursor peptide SSX-2_{37-58} encompassing the sequence of SSX-2_{41-49} was added directly to T2 cells (●). In parallel, the precursor peptide was incubated with proteasome for 0 (▲) or 30 (■) min at 37°C; the reaction was stopped by addition of 2% TFA; and samples were lyophilized, resuspended in medium to a concentration of 1 μM of the precursor peptide at time 0, and added in 3-fold dilution steps to T2 cells. *B,* Recognition of transfected COS-7 cells. Cells transfected with plasmids encoding HLA-A2, SSX-2, or SSX-4 (as indicated) were cocultured with effector cells at an E:T ratio of 2.5:1. The production of IFN-γ was measured by ELISA.

multimers but not by irrelevant multimers (Fig. 2*B*). The specificity of this clone was further confirmed in a ^{51}Cr release assay. It lysed T2 cells incubated with peptide SSX-2_{41-49} (with an apparent avidity of ~10 pM) but not with an irrelevant peptide (Fig. 3*A, left*). Decapeptide SSX-2_{40-49} was also recognized with an apparent avidity of ~100 pM. Whether peptide SSX-2_{40-49} binds to HLA-A2 and is recognized by CTLs as such or is first converted to SSX-2_{41-49} by degradation by serum proteases was not determined.

Because in vitro proteasome digestion experiments showed that the C terminus of peptide SSX-2_{41-49} could be generated, we tested whether the products generated by the proteasomal digestion of peptide SSX-2_{37-58} could sensitize T2 cells to lysis by the CTL clone (Fig. 3*A*). The undigested peptide SSX-2_{37-58} was recognized by clone LAU 50 E2.4 only at a very high concentration. In contrast, the same peptide preparation was recognized 100 times more efficiently after a 30-min incubation with proteasome, showing that proteasome contributed to the formation of the CTL clone-defined epitope.

Although these data showed that CTL could recognize a product generated by the proteasome in vitro, it was crucial to assess whether SSX-2_{41-49}-specific T cells were also able to recognize endogenously produced A2/SSX-2_{41-49} complexes. To this end, COS-7 cells were transiently transfected with plasmids encoding HLA-A2 and either SSX-2 or SSX-4 and tested for their capacity to induce IFN-γ production by SSX-2_{41-49}-specific clone LAU 50 E2.4. As shown in Fig. 3*B,* the CTL clone produced high levels of IFN-γ in the presence of cells cotransfected with plasmids encoding HLA-A2 and SSX-2 but not HLA-A2 and SSX-4, a gene homologue to SSX-2 but which differs by 2 aa within the antigenic peptide region. Untransfected cells or cells expressing either HLA-A2 or SSX-2 alone were not recognized.

To further verify the relation between the expression of SSX-2 and HLA-A2 and the recognition by SSX-2_{41-49} specific T cells, we used a panel of melanoma cell lines that were characterized for SSX-2 expression by RT-PCR (Fig. 4*A*) and HLA-A2 expression by Ab staining and flow cytometry analysis (data not shown). The cell lines were tested for recognition by anti-SSX-2_{41-49} clone LAU 50 E2.4 (Fig. 4*B*). SSX-2^+ and $A2^+$ autologous (Me 275) and allogeneic (SK-MEL-37) tumor cell lines were efficiently lysed by the CTL clone, in both the absence and presence of exogenously added peptide. SSX-2^- $A2^+$ SK-MEL-23 cells were not lysed unless peptide SSX-2_{41-49} was added exogenously. Finally, SSX-2^+ $A2^-$ Me 260 cells were not lysed by the CTL clone, neither in the presence nor in the absence of synthetic peptide.

Similar results, in terms of peptide recognition as well as recognition of transfected cells and tumor cell lines, were obtained with two other independent CTL clones (having different TCR β-chain variable domains as assessed by mAb staining and flow cytometry analysis) derived from the same TILN (data not shown). These results clearly demonstrate that a spontaneous T cell response against SSX-2 can take place in cancer patients and identify SSX-2_{41-49} as an HLA-A2-restricted $CD8^+$ T cell epitope.

Discussion

In the current study, we combined a proteasome-assisted epitope prediction approach with the screening of $CD8^+$ T cells derived from a tumor lesion to investigate the HLA-A2-restricted T cell immune response against the SEREX-defined CT Ag SSX-2 (HOM-MEL-40). This approach allowed us to identify the first

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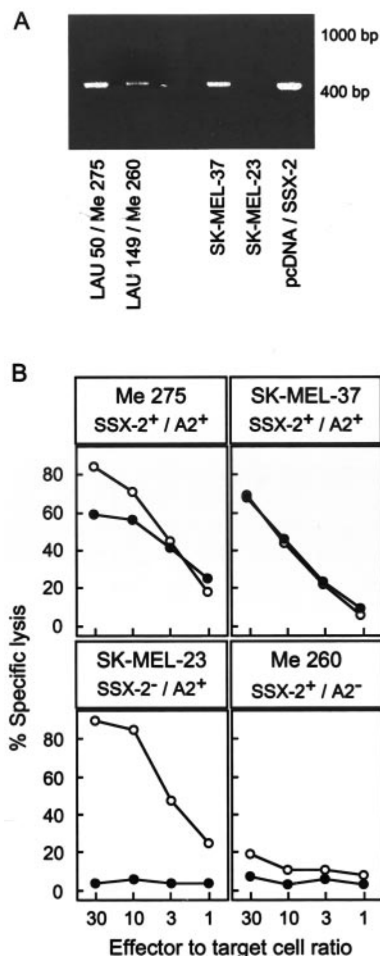

FIGURE 4. Lytic activity of SSX-2_{41-49}-specific clone LAU 50 E2.4 against melanoma cell lines. *A,* mRNA expression of SSX-2 in melanoma cell lines. RT-PCR was performed as described in *Materials and Methods*. SK-MEL-37 cells had already been shown to express SSX-2 and SK-MEL-23 cells had been shown not to express it (12). pcDNA plasmid containing SSX-2 cDNA was used as a positive control. *B,* Chromium-labeled target cells were incubated with effector cells at indicated E:T ratios in the absence (●) or presence (○) of SSX-2_{41-49} synthetic peptide (0.1 μM). HLA-A2 expression was assessed by mAb staining and flow cytometry analysis (data not shown).

SSX-2-derived $CD8^+$ T cell epitope and to show that a spontaneous $CD8^+$ T cell response against this epitope can occur in melanoma patients.

The SSX-2 gene was cloned as encoding a target of IgG Abs from a melanoma patient in the first SEREX study in 1995. It was also identified as one of the two genes involved in the translocation t(X;18)(p11.2;q11.2) observed in synovial sarcomas in 1994 (6, 7, 11). Expression analysis performed by Northern blot and RT-PCR demonstrated the presence of SSX-2 transcripts in a significant proportion of human cancers, including melanomas (35%), head and neck cancers (35%), lymphomas (36%), and colon carcinomas (12%), but not in normal tissues with the exception of testis (12, 22). In contrast to synovial sarcoma, no translocation implicating SSX-2 was observed in those malignancies. Anti-SSX-2 Abs were found in 10 of 89 patients with melanoma but not in 41 apparently healthy controls (7). Here we demonstrate for the first time that SSX-2 can elicit a spontaneous $CD8^+$ T cell response in addition to Abs. This response was detected in short term-cultured TILN of a patient with malignant melanoma who developed an anti-SSX-2 humoral response and whose tumor expressed SSX-2 mRNA. However, the correlation among seropositivity, SSX-2 expression in tumors, and T cell responses remains to be investigated. Indeed, in the case of NY-ESO-1, the only other SEREX-defined Ag for which a cellular immune response has been reported, $CD8^+$ T cell responses were found in virtually all seropositive patients tested but also in a smaller proportion of seronegative patients (10). SSX-2 is a member of the SSX multigene family composed of 5 genes the products of which share 77–91% sequence homology (12). All SSX genes are expressed in testis but not in other normal tissues, and all, with the exception of SSX-3, were found to be expressed in tumors (12, 22). Based on their high degree of homology, it would be of interest to study the immunogenicity of the other members of the SSX family that are expressed in tumors. *SSX-1* and *SSX-2* genes are both implicated in the translocation t(X;18)(p11.2;q11.2) observed in synovial sarcomas. This translocation event results in the expression of a fusion protein containing the 78 C-terminal amino acids of the SSX polypeptide (27), thus excluding the CTL epitope identified in our study. Whether this fusion protein is immunogenic and can therefore represent a potential target for immunotherapy of synovial sarcomas is unknown.

The original method used to identify peptide tumor Ags relied on the isolation of autologous tumor-specific CTL from cancer patients. These CTL were then used to screen Ag-negative target cells either transfected with cDNA libraries derived from tumor cells or pulsed with peptides eluted from MHC class I molecules purified from tumor cells (1, 2, 28). Because CTL used to identify these epitopes are tumor reactive, the antigenicity and proper processing of such epitopes are not questionable. More recently, a new strategy has been tested. This strategy, named reverse immunology, allows prediction, in silico, of the potential HLA class I ligands from any protein sequence based on the identification of appropriately positioned anchor residues specific for a given HLA class I allele (13, 20). The peptides that were predicted to bind to a given HLA class I molecule with the highest affinity were selected, and their immunogenicity was tested. Although this method allowed the identification of a number of promising peptides, several of the latter were not produced by tumor cells as demonstrated by the poor recognition of cells expressing the corresponding protein of interest endogenously (15–17). Hence, a crucial step for a correct application of this latter method is the demonstration that the generated CTL can recognize target cells expressing the antigenic protein. Regarding peptide SSX-2_{41-49}, the accumulation of specific T cells within melanoma lesions strongly supported the antigenicity and immunogenicity of the peptide. Moreover, the fact that specific CTL clones derived from TILN, which recognized the peptide with high apparent avidity, were able to recognize the endogenously produced epitope in tumor cell lines and in transfected COS-7 cells further confirmed the adequate production of the epitope by the cellular processing machinery. In contrast to the approach recently applied by Kessler et al. (18), in which they narrowed the number of high affinity HLA-A2 ligands derived from the tumor Ag PRAME by performing in vitro proteasomal digestions of peptides encompassing candidate peptides, we took the inverse approach, namely to identify among the peptides with C termini that were generated by proteasome those that were predicted to bind HLA-A2 with high or intermediate affinity. Because the C termini of >50% of the nonamers and 65% of decamers predicted to bind HLA-A2 with high or intermediate affinity were not generated by the proteasome in vitro, we were able to restrict the synthesis of peptides to be tested. In addition, our approach allows the simultaneous identification of potential ligands for various HLA class I molecules.

The majority of mammalian cells contain the standard proteasome. However, lymphoid cells (including myeloid-derived

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dendritic cells) or cells exposed to IFN-γ express the immunoproteasome, which differs from the standard proteasome by the presence of different catalytic subunits. Evidence indicates that such exchange results in a reduced production of several peptide tumor Ags (29, 30). Whereas digestion of PRAME peptides was performed using immunoproteasome (18), we used standard proteasome to digest the SSX-2 precursor peptides. Because the tumor cell environment may differ in terms of IFN-γ concentrations, it is possible that the proteasome composition and, consequently, the type of peptide tumor Ags produced may vary. However, preliminary experiments show that tumor cells cultured in presence of IFN-γ are recognized by SSX-2_{41-49} specific CTL clones, suggesting that this peptide can be generated by both standard and immunoproteasomes.

Peptide SSX-2_{41-49} is a new candidate peptide for immunotherapy of a wide range of human malignancies. However, the immunogenicity of this peptide in cancer patients (with malignant melanoma and other malignancies) and the efficiency of the peptide-induced CTL to recognize tumor cells should be determined before introducing peptide KASEKIFYV into clinical trials. Peptides homologous to SSX-2_{41-49} in the other SSX proteins differ by only 1 or 2 aa from peptide KASEKIFYV. We are currently investigating whether peptides derived from the same region of other SSX family members could also constitute T cell epitopes. In the case of MAGE-A1 and MAGE-A3 tumor Ags, two homologous but not identical peptides derived from the same region (MAGE-$A1_{160-168}$ and MAGE-$A3_{168-176}$) were shown to constitute two independent HLA-A1-restricted epitopes that stimulate distinct CTL repertoires (31). The other potential SSX-2-derived epitopes predicted in this study were not recognized by TILN from patient LAU 50. We are currently investigating the recognition of these peptides by TIL(N)s from other cancer patients.

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