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A Novel HLA-A*3303-Restricted Minor Histocompatibility Antigen Encoded by an Unconventional Open Reading Frame of Human TMSB4Y Gene

Hiroki Torikai,²,‡ Yoshiki Akatsuka,³,‡ Mikinori Miyazaki,*, Edus H. Warren III,⁧ Taku Oba,† Kunio Tsujimura,*, Kazuo Motoyoshi,‡ Yasuo Morishima,‡ Yoshihisa Kodera,† Kiyotaka Kuzushima,*, and Toshidata Takahashi*

Female-to-male hematopoietic stem cell transplantation (HSCT) elicits T cell responses against male-specific minor histocompatibility (H-Y) Ags encoded by the Y chromosome. All previously identified H-Y Ags are encoded by conventional open reading frames, but we report in this study the identification of a novel H-Y Ag encoded in the 5'-untranslated region of the TMSB4Y gene. An HLA-A*3303-restricted CD8+ CTL clone was isolated from a male patient after an HSCT from his HLA-identical sister. Using a panel of cell lines carrying Y chromosome terminal deletions, a narrow region controlling the susceptibility of these target cells to CTL recognition was localized. Minigene transfection and epitope reconstitution assays identified an 11-mer peptide, EVLLRPGLHFR, designated TMSB4Y/A33, whose first amino acid was located 405 bp upstream of the TMSB4Y initiation codon. Analysis of the precursor frequency of CTL specific for recipient minor histocompatibility Ags in post-HSCT peripheral blood T cells revealed that a significant fraction of the total donor CTL response in this patient was directed against the TMSB4Y epitope. Tetramer analysis continued to detect TMSB4Y/A33-specific CD8+ T cells at least up to 700 days post-HSCT. This finding underscores the in vivo immunological relevance of minor histocompatibility Ags derived from unconventional open reading frame products. The Journal of Immunology, 2004, 173: 7046–7054.

Minor histocompatibility (minor H) Ags are MHC-bound peptides derived from cellular proteins and are encoded by polymorphic genes, including Y chromosome-specific genes (1–3). Disparities in some minor H Ags in allogeneic hematopoietic stem cell transplantation (HSCT) have been shown to be associated with graft-vs-host disease (GVHD), graft rejection, or graft-vs-leukemia/lymphoma (GVL) effect (4–11). In the case of female to male HSCT, T cell clones specific for Y chromosome-encoded (H-Y) Ags were generated from the peripheral blood of recipients during GVHD or graft rejection, and their HLA class I or II epitopes have been identified, including SMCY (12, 13), DFFRY (14, 15), UTY (16, 17), RPS4Y (18), and DBY (19, 20). These five genes are among eight genes that have been reported to lie in the nonrecombining region of the human Y chromosome and have functional X homologues (21). Because all eight genes are sufficiently polymorphic with their X chromosome homologues to induce H-Y-specific T cell responses, it should be possible that more H-Y epitopes can be encoded either by the five genes that have proved to be immunogenic or by other Y chromosome genes (i.e., ZFY, AMELY, and TMSB4Y) for which H-Y epitopes have not yet been described.

In this study we report the identification of a novel human H-Y Ag, recognized by an HLA-A*3303-restricted CTL clone isolated from a male patient who developed chronic, but not acute, GVHD. The identified H-Y Ag is an 11-mer peptide, EVLLRPGLHFR, derived from TMSB4Y, a gene encoding thymosin β-4, isoform (22). Interestingly, the epitope identified in the TMSB4Y gene was encoded by the polymorphic region located 405 bp upstream of the initiation codon of the conventional open reading frame (ORF), whereas all minor H Ags identified to date are encoded by conventional ORF of the individual gene. There have been several reports describing CTL epitopes encoded by unconventional ORFs, such as untranslated regions (UTR) or alternative reading frames, most of which have been identified in tumor cells (reviewed in Ref. 23). To our knowledge, this is the first demonstration of a minor H Ag encoded in a region other than conventional coding region. Furthermore, we demonstrated, by CTL precursor (CTLP) frequency analysis, that a significant fraction of the total donor CTL responses in this patient was directed against the TMSB4Y epitope, and that the precursor remained detectable up to

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Abbreviations used in this paper: minor H Ag, minor histocompatibility Ag; CI, confidence interval; CTLP, CTL precursor; DRIP, defective ribosomal product; GVHD, graft-vs-host disease; GVL, graft vs leukemia/lymphoma; HSCT, hematopoietic stem cell transplantation; H-Y Ag, Y chromosome-encoded Ag; LCL, B-lymphoblastoid cell line; ORF, open reading frame; UTR, untranslated region; STS, sequence-tagged site.
700 days after HSCT. These findings underscore the in vivo immunological relevance of such a cryptic minor HAg derived from unconventional ORF products.

Materials and Methods

Cell cultures and Abs

The HLA-A*0303-restricted CD8\(^+\) CTL clone, IB6, was isolated by limiting dilution from a cytotoxic T cell line generated from a PBMC sample obtained on day 50 post-HSCT from a 54-year-old man (HLA-A*2402+/*303, B*4403/*5401, Cw*0803/*1403) who had received his HLA-identical sister’s marrow for treatment of chronic myelocytic leukemia. He did not develop acute GVHD, but did develop mild chronic GVHD of the skin and liver. The CTL clone was expanded as previously described (24) and frozen until use. B-lymphoblastoid cell lines (LCLs) were established from the donor and recipient and from normal volunteers. All blood or tissue samples were collected after obtaining written informed consent, and the study was approved by the institutional review board of Aichi Cancer Center.

The LCLs derived from individuals with Y chromosome deletions were provided by Dr. D. C. Page (Howard Hughes Medical Institute, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA), and a detailed analysis of these lines has been previously reported (25). LCLs selected according to their deletion pattern and other cell lines including Raji were retrovirally transduced with HLA-A*0303 cDNA as described previously (26). LCLs were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (Immu-no-Biological Laboratory, Gunma, Japan), 2 mM t-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. Primary dermal fibroblast lines from skin and oral mucosa, bone marrow stromal cell lines, and 293T cells were grown in IMDM (In vitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FCS, 2 mM t-glutamine, and penicillin/streptomycin. mAbs, W6/32 (anti-pan HLA class I), HDR-1 (anti-HLA-DR), and A11.1M (anti-HLA-A24) were provided by Dr. K. Ito (Kurume University, Fukuoka, Japan).

Cytotoxicity assays

Target cells were labeled with 0.1 mcg of \(^{51}\)Cr for 2 h, and \(1 \times 10^6\) target cells/well were mixed with CTL at various E:T cell ratios in a standard 4-h cytotoxicity assay using 96-well, round-bottom plates. All assays were performed at least in duplicate. Cells were treated with IFN-\(\gamma\) (100 U/ml; Endogen, Woburn, MA) and TNF-\(\alpha\) (10 ng/ml; Endogen) for 48 h where indicated. The percent specific lysis was calculated as follows: (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) \(\times\) 100. When necessary, allo-HLA-A24-specific CTL clones were used to confirm the susceptibility of the target cells.

Mapping of Y chromosome deletion mutant LCLs

Oligonucleotide primer pairs specific for sequence-tagged sites (STSs) previously mapped to the Y chromosome (25) were used to PCR-amplify the corresponding Y chromosomeal target sequences from genomic DNA of each LCL. Amplification of STSs was performed as reported previously (16). Aliquots of each PCR were separated in 2% agarose or 5% acrylamide gels, and cell lines were scored as positive or negative for the presence of each STS. DNA extracted from LCLs derived from normal male and female donors served as positive and negative controls, respectively.

Detection of expression of the candidate genes

An RT-PCR assay was used to examine the expression of the candidate genes with cDNA synthesized from LCLs. PCR was performed in a total volume of 20 \(\mu\)l containing 1 \(\times\) PCR buffer, 1.5 mM MgCl\(_2\), 200 \(\mu\)M of each dNTP, 0.5 \(\mu\)g of each gene-specific primer, and 1 \(\mu\)l of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) on a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were separated in 2% agarose gels and visualized with ethidium bromide staining.

PCR cloning of TMSB4Y gene

The conventional ORF sequence and full-length sequence of the TMSB4Y (GenBank accession no. NM_000420) were amplified from cDNA prepared from the recipient LCL and subcloned into a mammalian expression plasmid. The primer sequences used were as follows (HindIII and NotI sites are underlined, respectively): conventional ORF sense, 5\'-TTAGCGCTCACCACCATGTCACTGCAACACC-3'; conventional ORF antisense, 5\'-ATGGCGCCCGCATCATGCTTTAAAAGTGCGGC-3'; full-length sense, 5\'-TTAAGCTTTGGGACACGACAGATCCCTTTG-3'; and full-length antisense, 5\'-ATGGCGCCCGCATCATGCTTTAAAAGTGCGGC-3'. PCR amplification was conducted in a total volume of 25 \(\mu\)l of 1 \(\times\) buffer containing 200 \(\mu\)M of each dNTP, 1.0 mM MgSO\(_4\), 0.3 \(\mu\)M of each primer, and 1 U of KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan).

All products were digested with the restriction enzymes and ligated into HindIII-Not1-cut pEAK10 vector (Edge Biosystems, Gaithersburg, MD). The sequences of the cloned genes were verified by direct sequencing with BigDye Terminator Kit (version 3.0, Applied Biosystems) on an ABI PRISM 3100 (Applied Biosystems).

Construction of truncated genes and minigenes for TMSB4Y

Expression plasmids encoding truncated forms of the TMSB4Y cDNA were constructed by RT-PCR using antisense primers that produced 345, 552, 754, and 955 bp DNA fragments. All products were ligated into the pEAK10 vector as described above. Minigene expression plasmids encoding the minimal, N or C terminus-extended polypeptides of the epitope predicted by BIMAS software were generously provided by D. H. Bert.nih.go.jp/shiba_bind (27) and SYFPEITHI software (http://syfpeithi.de) (28) were constructed as previously described (29). The constructs all encoded a Kozak sequence and initiator methionine (CCACC-ATG) and a stop codon (TAG). Pairs of sense and antisense oligonucleotides were designed to form cohesive ends for HindIII and NotI sites at the 5’ and 3’ ends after hybridization, respectively, and all products were ligated into the pEAK10 vector and verified by sequencing.

Electroporation of LCL

The constructed vectors were introduced either into the donor LCL or into 293T cells. One million LCLs were resuspended in 40 \(\mu\)l of OPTI-MEM 1 buffer (Invitrogen Life Technologies) and 4 \(\mu\)g of each plasmid in a 2-mm gap cuvette and electroporated in an ECM 830 BTX Electro Square Porator (BTX, San Diego, CA) at 350 V and a pulse length of 1 ms. Then, cells were cultured in 4 ml of culture medium for 2 d, followed by selection with puromycin (0.7 \(\mu\)g/ml) for 3 d before use.

Transfection of 293T cells and cytokine release assays

293T cells were retrovirally transduced with HLA-A*0303 cDNA and selected in the presence of 1 \(\mu\)g/ml puromycin (referred to as 293T-A33). Aliquots of the 293T-A33 cells were transiently cotransfected with pEAK10 vectors encoding full-length TMSB4Y, a C-terminal deletion mutant cDNA, or minigenes of TMSB4Y. 293T-A33 cells were plated the day before transfection at 4 \(\times\) 10\(^5\) cells/100 \(\mu\)l/well and 96-well, flat-bottom microtiter plates and transfected with 6 \(\mu\)l of RPMI 1640 containing 90 ng of plasmid DNA and 0.27 \(\mu\)l of FuGENE 6 (Roche, Indianapolis, IN). After transfection at 37°C, 100 \(\mu\)l of a cell suspension containing 1 \(\times\) 10\(^5\) cells per clone IB6 in IMDM containing 20 U/ml IL-2 was added. Supernatants from the cocultures were harvested after 24 h and assayed for the presence of IFN-\(\gamma\) by ELISA.

Epitope reconstitution assay

The candidate peptide epitope identified by the minigene experiments and the homologous TMSB4X-encoded peptide were synthesized by standard methods. \(^{35}\)S-labeled donor LCL were incubated for 30 min in medium containing 10-fold serial dilutions of the peptides and then used as target cells in standard cytotoxicity assays.

Real-time PCR assay for TMSB4Y expression

cDNA from a panel of different human adult and fetal tissues were purchased from BD Clontech (MTC panels human I and II; Palo Alto, CA) or synthesized from total RNA of human lung (BD Clontech) or various cultured cells. PCR amplification and real-time quantification analysis were performed using the TaqMan assay according to the manufacturer’s instructions. The following sequences were used as primers and TaqMan probe to detect the mRNA region encoding the epitope: 5’-GACTAGAACGGGCGCGCGGACG-3’ (sense; nt 302–320), 5’-ACTCTCGGCTTCAAGTGCTTT-3’ (antisense; nt 415–434), 5’-FAM-TCCCTTCTGACAGGAGCTCATATGTTGAGT-(MGB)-3’ (TMSB4Y probe; antisense; nt 366–382), for the internal control, a primer and probe set for human GAPDH (Applied Biosystems) was used. PCR was performed in a 1 \(\times\) TaqMan Universal PCR master mix containing 10 pmol of each sense and antisense primer and 2 pmol of probe in a total volume of 25 \(\mu\)l in the ABI PRISM 7700HT Sequence Detector System (Applied Biosystems). The temperature profile was 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 62°C for 1 min for 40 cycles. Samples were quantified using relative standard curves for each amplification. All results are normalized with
respect to the internal control and are expressed relative to the levels found in a pool of male PBMC.

Limiting dilution-based CTLp frequency assay

The proportion of CTLp specific for the TMSB4Y peptide among the total CTLp against the recipient minor H Ags was quantitated using a standard limiting dilution assay. Purified CD8⁺ T cells from the PBMC obtained at days 50 and 146 post-HSCT were cultured at 2-fold serial dilutions with 33 Gy-irradiated 3 x 10⁶ CD40-activated B (CD40-B) cells generated from pre-HSCT recipient PBMC in 96-well, round-bottom plates in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% pooled human serum. IL-2 (50 U/ml) was added on days 2 and 5 after each restimulation with CD40-B cells. For each dilution, there were at least 12 replicates. After three rounds of stimulation, a split-well analysis was performed for peptide-specific cytolysis against ³¹Cr-radiolabeled recipient PHA blasts or donor PHA blasts pulsed with TMSB4Y peptide or unpulsed. The supernatants were measured in a gamma counter after 4-h incubation. The wells were considered to be positive for lytic activity if the total cpm released by effector cells was >2.5 x SD above control wells (mean cpm released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated by L-Calc software (StemCell Technologies, Vancouver, Canada).

Tetramer construction and flow cytometric analysis

MHC-peptide tetramers were produced as described previously (30). In brief, HLA-A*3303 H chain and β₂-microglobulin (cloned in pHLN1 vector; provided by the late Dr. D. C. Wiley, Howard Hughes Medical Institute, Harvard University, Cambridge, MA) were produced in pX90. The C terminus of the H chain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA/β₂-microglobulin/peptide complexes were folded in vitro in the presence of the peptide. The HMC complex was biotinylated and then converted into tetramers with PE-labeled streptavidin. For staining, PBMC or T cell lines were incubated with the tetramer at a concentration of 20 μg/ml at room temperature for 15 min, followed by FITC-conjugated anti-CD3 (BD Biosciences, San Diego, CA) and Tricolor anti-CD8 mAb (Caltag Laboratories, Burlingame, CA) on ice for 15 min. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Results

A CD8⁺ CTL clone shows cytotoxicity against an H-Y Ag presented on HLA-A*3303⁺ LCL

CD8⁺ CTL clone 1B6 efficiently lysed recipient LCL and PHA blasts, but not donor LCL (Fig. 1A). Addition of anti-pan HLA class I mAb, but not anti-HLA-A24 or anti-HLA-DR mAbs, significantly inhibited lysis of recipient LCL by 1B6. Transduction of HLA-A*3303 cDNA into a male LCL conferred susceptibility to 1B6, indicating that the clone was restricted by HLA-A*3303 (Fig. 1B). 1B6 showed very weak cytotoxicity against dermal or oral fibroblasts or against keratinocytes generated from HLA-A*3303-positive male individuals, whereas these targets were lysed moderately (i.e., 25-35%) by CTL specific for HLA-A24 alloantigen, which is shared by these targets. Even when they were treated with cytokines (IFN-γ and TNF-α), 1B6 still demonstrated relatively weak cytotoxicity, although HLA-A24-allopecific CTL induced robust cytotoxicity (Fig. 1C). Finally, 1B6 showed lytic activity only against male, but not female, LCLs transfected with HLA-A*3303 cDNA, indicating that the clone was specific for an H-Y Ag (data not shown).

The gene encoding the minor H Ag maps to deletion interval 5D on the Y chromosome

Cytotoxicity assay-based mapping was conducted to determine the location on the Y chromosome of the minor H gene encoding the epitope for 1B6. First, various LCLs were typed for terminal deletions of the Y chromosomes using the technique of STS content mapping (16, 21). Of these, a panel of LCLs with distinct terminal deletions was selected for transfection with HLA-A*3303 cDNA and assayed for susceptibility to 1B6.

Fig. 2A shows the 43-interval deletion map of the 7 LCLs and their susceptibility to 1B6. LCL WHY10 and WHY12 that were lysed by 1B6 share only deletion intervals 5C and 5D, indicating that the region controlling the expression of this minor H Ag is...
located within these two deletion intervals. The region was further narrowed down using the results from LCL WHY24, which was found to lack deletion interval 5C, but was nevertheless lysed by 1B6. Collectively, these results indicate that the gene encoding the minor H Ag maps to deletion interval 5D. Four genes, DBFY, DBY, UTY, and TMSB4Y, all of which have X homologues, are encoded within deletion intervals 5C and 5D (21, 25). We examined mRNA expression of these four genes among the seven LCLs by RT-PCR (Fig. 2A). As expected from the results of deletion mapping, WHY6 and WHY11 were negative for the expression of all four genes; WHY10, WHY9, WHY17, and WHY12 were all positive. Because WHY24 was positive for the expression of TMSB4Y and UTY and was lysed by 1B6, the minor H Ag was encoded by either UTY or TMSB4Y. In addition, during the course of specificity analysis, we found that HLA-A*3303-transduced Raji cells were not killed by 1B6, although Raji cells are of male origin. RT-PCR analysis showed that they were negative for expression of TMSB4Y as shown in Fig. 2B. Moreover, female LCLs from the patient’s HSCT donor transfected with any of three isoforms of UTY cDNA were not lysed by 1B6 (data not shown). These results indicated that TMSB4Y most likely encoded the minor H Ag.

The 5’ untranslated region of the TMSB4Y gene encodes the minor H Ag

To determine whether TMSB4Y indeed encodes the minor H Ag epitope recognized by 1B6, we first tested CTL recognition of the female donor LCL transduced with the reported TMSB4Y ORF comprising 43 aa. However, 1B6 did not lyse the transfectant (Fig. 3A), suggesting either that the epitope is encoded not by TMSB4Y but by another gene located in deletion interval 5D, or that it is encoded elsewhere in the ~1.7-kb TMSB4Y cDNA. Recently, cryptic CTL epitopes encoded by alternative sources such as non-coding regions and nonconventional ORF have been described in both murine and human tumor cells (23). Thus, we cloned the full-length TMSB4Y cDNA (GenBank accession no. NM_004202) and then transduced donor LCL with it. As shown in Fig. 3B, female LCL expressing full-length TMSB4Y cDNA were lysed efficiently. Because the alternative ORF that is able to encode the antigenic peptide was unknown, a series of 3’ terminal deletion mutants of the TMSB4Y cDNA were prepared and tested for recognition by 1B6 by IFN-γ ELISA. Although cells transfected with TMSB4Y cDNA fragments extending from nt 1–552 were recognized when expressed in HLA-A*3303-transduced 293T cells, transfection of the fragment extending nt 1–345 was not (Fig. 3C). These results indicated that the epitope was encoded in the 5’UTR between nt 346 and 552, which is at least 240 nt upstream of the reported ORF for the TMSB4Y protein (Fig. 4A).

Among three reading frames in this region, only one initiator methionine (nt 362–364) was found in the same reading frame encoding the TMSB4Y protein, followed by a polypeptide consisting of 19 aa, EVLLRPGLHFRNSCPILTT. This 19-mer contains a nonamer, LLRPGLHFRNSCPILTT, which has the reported peptide-binding motif for HLA-A*3303 (i.e., Ala, Ile, Leu, Phe, Tyr, or Val at position 2, and Arg at C terminus) (31), with a predicted dissociation score of 9.0 by BIMAS software (27). However, a minigene construct encoding LLRPGLHFR failed to stimulate 1B6. Additional experiments using minigene constructs with N or C extensions finally identified the minimal epitope as EVLLRPGLHFRNSCPILTT. This 19-mer contains a nonamer, LRLRPGPLHFR, which has the reported peptide-binding motif for HLA-A*3303 (i.e., Ala, Ile, Leu, Phe, Tyr, or Val at position 2, and Arg at C terminus) (31), with a predicted dissociation score of 9.0 by BIMAS software (27). However, a minigene construct encoding LRLRPGPLHFR failed to stimulate 1B6. Additional experiments using minigene constructs with N or C extensions finally identified the minimal epitope as EVLLRPGLHFR (Figs. 3D and 4A). Both Arg at the C terminus and Glu at the N terminus were essential for recognition by 1B6, indicating that Val and Arg are the likely N- and C-terminal anchors, respectively. The X homologue of TMSB4Y, TMSB4X cDNA (GenBank accession no. NM_021109), encoding thymosin β4, has much shorter 5’- and 3’UTR; thus, no corresponding region was found (Fig. 4B). However, a recently reported splice variant of TMSB4X, which includes 1076 bp of TMSB4X intron 1 (GenBank accession no. AK055976), has an initiator methionine and a following 32 aa in its 5’UTR upstream TMSB4X conventional ORF, and potentially encodes ETTLFLPLHFR, which differs from the 1B6

Figure 2. Mapping of the gene on the Y chromosome that encodes the minor H Ag recognized by 1B6. A, Genetic map of the LCLs carrying various terminal deletions of Y chromosome and their susceptibility to 1B6. Appropriate LCLs were selected based on their pattern of terminal deletions (16, 21, 25), transduced with HLA-A*3303 cDNA, and tested in standard 51Cr release assays. The presence of the region encoding the minor H Ag in each LCL line is determined by its susceptibility to 1B6 (indicated in the right column). Vertical bars indicate the region predicted to encode the minor H Ag. B, mRNA expression of four genes encoded in the deletion intervals 5C and 5D in selected cell lines and their recognition by 1B6. Female donor LCL served as a negative control. HLA-A*3303 transfected Raji cells, a Burkitt lymphoma cell line derived from a male patient, were also analyzed.
and assayed for the presence of IFN-552, 754, or 955 nt and cocultured with 1B6. Supernatants were harvested.

Accession no. NM_004202) was transfected into donor LCL, and their susceptibility to 1B6 was tested as described above. The assays clearly detected TMSB4Y/A33-specific CD8+ T cells in PBMC obtained on day 696 (0.35%), but for PBMCs obtained on day 50 and 146, the presence of TMSB4Y/A33-specific T cells was not detectable.

TMSB4Y/A33-specific CD8+ T cells are detectable in recipient post-transplant PBMC

A split-well assay was used to estimate the relative frequencies in the post-HSCT PBMC of CTLp specific for the TMSB4Y/A33 minor H Ag and those specific for other minor H Ags expressed on the recipient’s hemopoietic cells. As shown in Fig. 6A, the frequencies of CTLp reactive with recipient PHA blasts and TMSB4Y/A33 peptide-pulsed donor PHA blasts in peripheral blood obtained on day 50 post-HSCT from which the 1B6 was derived were 324 (95% confidence interval (CI), 213–493) per 10^6 peripheral blood CD8+ cells, respectively, indicating that nearly a quarter of the CTL responses to recipient minor H Ags in this donor/recipient pair were indeed directed at the TMSB4Y/A33 minor H Ag. On day 146, the frequency of CTLp recognizing TMSB4Y peptide-pulsed donor PHA blasts was 316 (95% CI, 216–464), and that for CTLp recognizing recipient PHA blasts was 3215 (95% CI, 2150–4808) per 10^6 peripheral blood CD8+ cells, demonstrating that even at the later time point the CTL responses against TMSB4Y/A33 continued to account for a significant fraction (10%) of the total donor CTL responses against recipient minor H Ags in this donor/recipient pair (Fig. 6B).

In additional experiments, an HLA/peptide tetramer was used to confirm the presence of TMSB4Y/A33-specific CTL in unstimulated post-HSCT PBMC (Fig. C, left column) as well as in T cell lines prepared by stimulating these PBMC with the same stimulators used in the CTLp assay (Fig. C, right column). The assays were readily detected TMSB4Y/A33-specific CD8+ T cells in PBMC obtained on day 696 (0.35%), but for PBMCs obtained on day 50 and 146, the presence of TMSB4Y/A33-specific T cells was not clear because of the low number of PBMC available. After in vitro stimulation, tetramer-positive cells became detectable for the latter two samples, although direct comparison with the CTLp results was not possible due to the use of different culture conditions in the two assays.

Discussion

In this study we have identified a gene, TMSB4Y, encoding a novel HLA-A*3303-restricted, H-Y Ag by testing HLA-A*3303-transfected cell lines carrying terminal deletions of the Y chromosome in cytotoxicity assays. This approach has previously been used to identify the HLA-B8-restricted H-Y Ag encoded by UTY (16).
With the current discovery, all four identified genes that are encoded in deletion intervals 5C and 5D of the nonrecombining region of the human Y chromosome have been shown to encode at least one minor H Ag presented by class I or II HLA (14–17, 19, 20). Although the peptide sequence of the TMSB4Y/A33 minor H Ag identified in this study was 11 residues in length, and half-maximal lysis of peptide-pulsed female target cells was observed at a relatively high peptide concentration (≈20 nM), it is likely that the 11-mer peptide is the minimal epitope, because it has a consensus Arg at the C terminus and a Val at the auxiliary anchor (position 2) (31). In addition, two computer algorithms predict that cleavage after the C-terminal Arg would be correctly performed by proteasomes (33, 34). Although all previously identified human H-Y Ags have homologue peptide on the ORF of their X homologous gene, it is not yet clear whether TMSB4Y/A33 minor H Ag has its homologue, because the longest cDNA clone (GenBank accession no. AK055976) assigned to be one of the splice variants of the TMSB4X gene containing the first intronic sequence was not detected by RT-PCR, whereas the full-length cDNA encoding thy- 

mosin β4 was readily detectable (data not shown). Thus, it is conceivable that the splice variant, AK055976, might be very rare or derived from a precursor mRNA.

Recently, evidence has been accumulating that cryptic polypeptides derived from noncoding regions, such as UTRs or introns, or encoded in alternative ORFs occasionally encode CTL epitopes for tumor or viral Ags in humans or mice (reviewed in Ref. 23). Of these, only one epitope is found in the 5′UTR of a cellular oncogene, c-akt, in the murine RL.Δ1 leukemia system (35). This unusual epitope is generated by insertion of the murine leukemia virus long terminal repeat into the exon of c-akt, resulting in transcription initiated at the cap site of the long terminal repeat. To the best of our knowledge, this is the first demonstration of a minor H Ag encoded outside a conventional ORF of a nonmutated gene. Although it is possible that the 19-residue ORF in the 5′UTR that encodes the epitope is an as yet unrecognized functional coding region, a search of the protein database, including the Protein-Protein Blast (http://www.ncbi.nlm.nih.gov/blast/), for amino acid sequence homology to this region did not identify any known functional domains.
Moreover, when the whole TMSB4Y genomic region was analyzed using GENESCAN software (36) (http://genes.mit.edu/GENESCAN.html), no ORF other than the reported ORF encoding the 34-mer polypeptide was predicted with a risk of <1.6% of false negative. These results strongly suggest that the TMSB4Y/A33 minor H Ag is not derived from a functional polypeptide, but, rather, that it is a subsidiary translation product of the TMSB4Y transcript.

Defective ribosomal products (DRiPs) consist of prematurely terminated polypeptides and misfolded polypeptides produced from translation of genuine mRNAs in the proper reading frame or are produced entropically due to the inevitable imperfections inherent to protein synthesis or folding (37). DRiPs, which account for 30% of newly synthesized proteins, have been suggested to be a major source of peptides presented on the cell surface by class I MHC (38). TMSB4Y was expressed in normal cells as well as transformed cells when assessed by quantitative PCR specific for the region encoding the TMSB4Y/A33 epitope, and the full-length mRNA was readily detected (data not shown). According to the definition of DRiPs, which is defective products from genuine mRNAs in the proper reading frame, TMSB4Y/A33 should be one of epitopes derived from cryptic polypeptides rather than DRiPs. In any case, the identification of a minor H Ag encoded outside the conventional ORF has important implications for the identification of other minor H Ag epitopes using genetic linkage analysis. Recently, we identified two minor H Ags using a similar approach (29), where we looked for peptides with potential HLA-binding sequence motifs that spanned nonsynonymous single nucleotide polymorphisms in the conventional ORF. However, the results of the current study suggest that not only conventional ORFs but also regions other than conventional ORFs should be taken into consideration when attempting to identify the epitope within the region mapped by linkage analysis.

Although the function of TMSB4Y is not yet known, its X chromosome homologue, TMSB4X, also known as thymosin β4, encodes a protein that plays an important role in the organization of the cytoskeleton, which binds to and sequesters actin monomers (G actin), leading to inhibition of actin polymerization (22). As expected from its function, thymosin β4 is highly expressed in metastatic melanoma cells together with fibronectin and RhoC, a member of the Rho GTPase family (39). Because Rho-like GTPases are suggested to be linked with HA-1 (40) and HA-3 (41) proteins in cytoskeleton rearrangement and have myosin 1G encoding HA-2 minor H Ag (42) as one of the downstream effector proteins (43), TMSB4Y/A33 derived from the Y homologue of thymosin β4 may also be classified as one of malignancy-associated minor H Ags according to the recent proposal by Spierings et al. (43).

CTLp frequency assays revealed that the magnitude of the CTL response to the TMSB4Y/A33 epitope was early after HSCT and represented one-quarter of the measurable donor CTL responses to
recipient H Ags in this donor/recipient pair. This illustrates the extent to which such cryptic peptides may contribute to the diversity and immunogenicity of the total class I MHC-associated peptide pool in normal cells. In this regard, the relative immunogenicity of another minor H Ag, HB-1, which is derived from a polypeptide whose translation is initiated at a CUG instead of a conventional ATG codon exclusively in transformed B cells (44, 45), should also be of interest. Recently, Schwab et al. (46) have shown that the insertion into a 3′UTR of a sequence encoding an antigenic peptide elicits T cells specific for this peptide in vivo, which recognize at least DCs, B cells, and fibroblasts from mice carrying the transgene. In contrast, analysis of >200 endogenously derived HLA-B*1801-associated peptides from a human B cell line revealed that all the peptides were encoded by conventional ORFs from a wide variety of cellular genes (47), suggesting that the frequency of cryptic peptides being presented on class I MHC molecules is <1/200. Identification of more minor H Ags may answer the question of the significance of cryptic peptides over molecules.

A recent study has suggested that CTL responses against minor H Ags encoded or regulated by genes on the Y chromosome conventional peptides. The answer to elucidate whether this cryptic product can serve as a target for immunotherapy against hematological malignancies is unknown. Hiromi Tamaki for their expert technical assistance.

**References**


