Preparation of APC

DC were prepared as previously described with slight modification [51]. Briefly, $CD8^+$ T cells were isolated from PBMC using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and stored at –135°C until use. The CD8-depleted PBMC were suspended in 4 mL RPMI 1640 medium supplemented with 5% human serum (MP Biomedicals, Aurora, OH), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin (referred to as DC medium), and incubated for 2 h in wells of 6-well plates at 37°C. Nonadherent cells were removed by gentle pipetting, and the adherent cells were cultured in DC medium in the presence of 50 ng/mL GM-CSF (Osteogenetics, Wuerzburg, Germany) and 10 ng/mL IL-4 (Osteogenetics). On days 2 and 4, half of the medium was replaced with fresh DC medium containing GM-CSF and IL-4. On day 6, DC were collected and electroporated for mRNA transduction. CD40-B cells were generated as described previously [50, 52] using NIH/3T3 human CD40 ligand cells (kindly provided by Dr. Freeman, Dana-Farber Cancer Institute, Boston, MA).

Plasmid construction

To construct ΔLMP1 [20], PCR was performed using a sense primer 5′-aagcttgccaccATGAGTGACTGGACTGGA-3′, an antisense primer 5′-ttgaattcttagtcatagtagcttagctga-3′, and EBV strain B95-8 (NCBI accession No. V01555) cDNA as a template. The resultant DNA fragment was cloned into pcDNA 3.1(+) (Invitrogen, Carlsbad, CA) using its *Hind*III and *Eco*RI sites (pcDNA/ΔLMP1). For constructing further C-terminal and N-terminal deletion mutants of the ΔLMP1 gene, truncated fragments were prepared by PCR using pcDNA/ΔLMP1 as a template, and cloned into the pcDNA3.1(+). To construct some plasmids encoding short LMP1 peptide fragments, each pair of complementary oligonucleotides were annealed and cloned into restriction enzyme-cut pcDNA3.1(+). ΔLMP1 and EGFP [50] cDNA were cloned into the pMSCVpuro retroviral vector (BD Biosciences Clontech, Palo Alto, CA) to generate pMSCVpuro/ΔLMP1 and /EGFP, respectively.

shRNA interference retrovirus vectors were constructed using the RNAi-Ready pSIREN-RetroQ vector (BD Biosciences Clontech). The following siRNA targets were selected in this study: ip-LMP2, AAGUGAAGGAGGUCAGGUAUA, and ip-LMP7, AGAUUAACCCUUACCUGCUTT. The shRNA constructs included a TTCAAGAGA loop separating the sense and antisense sequences followed by a 5T termination signal. These constructs were synthesized as two complementary DNA oligonucleotides, annealed, and ligated between the *Bam*HI and *Eco*RI sites of the vector. In addition, a negative control siRNA annealed oligonucleotide (BD Biosciences Clontech) was inserted into the same vector and used as a control. The cloned genes were sequenced to verify their identity.

Retrovirus production and infection

To establish retrovirus-producing cells, pMSCVpuro/ΔLMP1, pMSCVpuro/EGFP and RNAi-Ready pSIREN-RetroQ-based vectors were packaged in PT67 cells (BD Biosciences Clontech) using Lipofectamine 2000 (Invitrogen). LCL were infected with the retroviral supernatant in the presence of 8 μg/mL polybrene (Sigma Chemical Co.), spun at 1000 × g at 32°C for 1 h, and incubated at 37°C for 2 days. Thereafter, these LCL were cultured in the presence of 0.8 μg/mL puromycin for 14 days. Expression of EGFP and ΔLMP1 was analyzed by FCM. Expression of ip-LMP2 and ip-LMP7 was assessed by Western blotting as described previously [53, 54].

Production and transduction of *in vitro* transcribed mRNA

Fragments containing the T7 promoter region and the ΔLMP1 coding region were prepared by PCR using pcDNA/ΔLMP1 as a template. The amplified DNA was used as a template for *in vitro* transcription of 5′-capped mRNA using a mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions. The 3′ polyA tail was added using polyA polymerase (Ambion) followed by purification with an RNeasy kit (QIAGEN, Tokyo, Japan).

Prior to electroporation, DC and CD40-B cells were washed twice with serum-free RPMI 1640 medium and suspended to a final concentration of 2.5×10^7 cells/mL. Cells in 40 μL were mixed with 20 μg of mRNA, and electroporated in a 0.2-cm cuvette using an Electro Square Porator ECM 830 (Harvard Apparatus, Holliston, MA). The conditions were 450 V and 500 μS for DC and 350 V and 350 μS for CD40-B cells. After electroporation, DC were cultured in DC medium supplemented with GM-CSF and IL-4 for 3-h, then exposed to TNF-α (PeproTech, Rocky Hill, NJ), IL-1β (PeproTech) and prostaglandin E2 (Cayman Chemical Company, Ann Arbor, MI) for maturation. CD40-B cells were immediately seeded onto irradiated NIH/3T3 human CD40 ligand cells, and after 36–48 h used as APC.

Cell staining and FCM

Intracellular staining of LMP1 Ag was performed as previously described with slight modification [20]. Briefly, electroporated cells were collected and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were permeabilized with IC Perm (BioSource International, Camarillo, CA) and reacted with an mAb recognizing the C terminus of LMP1 (CS1-4; DAKO Cytomation, Glostrup, Denmark) for 30 min at 4°C. After washing with PBS, cells were stained with fluorescein isothiocyanate-labeled anti-mouse IgG (H+L) (Immunotech, Marseille, France) for 30 min at 4°C. The stained cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA) and CellQUEST software (BD Biosciences).

CTL induction

Stored $CD8^+$ T cells were thawed, washed, and co-cultured with irradiated (33 Gy) autologous ΔLMP1 mRNA-transduced DC in 2 mL RPMI 1640 medium supplemented with 10% human serum, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin, in the presence of 25 ng/mL IL-7 (R&D Systems, Minneapolis, MN) and 5 ng/mL IL-12 (R&D Systems) at 5% CO_2 in a humidified incubator. On days 8 and 15, T cells were restimulated with ΔLMP1 mRNA-

transduced and γ-irradiated DC and CD40-B cells, respectively. One day after each restimulation, IL-2 (Shionogi, Osaka, Japan) was added to a final concentration of 20 U/mL. To establish T cell clones, limiting dilution of polyclonal CTL was performed using round-bottomed 96-well plates as previously described [55]. After 2 wk of culture, growing wells were split into three replicates and used as effectors in the CTL assay against either ΔLMP1 mRNA- or EGFP mRNA-transduced autologous CD40-B cells. Wells were scored as positive when the counts per minute from ΔLMP1 mRNA-transduced CD40-B cells exceeded the mean counts per minute from EGFP mRNA-transduced CD40-B cells by three standard deviations. Positive wells were transferred into flasks and expanded as previously described [55].

ELISPOT assay

ELISPOT assays were performed as described earlier [48, 50, 55]. Briefly, $CD8^+$ T cells were co-cultured with various stimulators in wells of Multiscreen-HA plates (MAHA S4510; Millipore, Billerica, MA) coated with anti human IFN-γ mAb (M700A; Pierce Biotechnology, Philadelphia, PA). As stimulators, HLA-A*0206-positive or -negative LCL (1×10^5 cells/well) or HLA-A*0206-expressing HEK-293T (referred to as A0206-293T) cells (5×10^4 cells/well) transfected with plasmids using Lipofectamin 2000 (Invitrogen) 48 h earlier were seeded into each well. For peptide titration assays, serial concentrations of synthetic peptides (Greiner, Frickenhausen, Germany) were pulsed to A0206–293T cells for 1 h at room temperature. After probing with anti-rabbit polyclonal IFN-γ antibody (P700; Pierce Biotechnology), followed by exposure to horseradish peroxidase-labeled anti-rabbit IgG antibody (Genzyme, Cambridge, MA) and spots visualization, the plates were washed and dried. IFN-γ spots were enumerated using a dissecting microscope.

CTL assay

Target cells were labeled with 50 μCi ^{51}Cr for 1.5 h at 37°C, washed, and mixed with CTL at the indicated effector-to-target ratios in 96-well plates. After incubation for 4 or 16 h at 37°C, the radioactivity in the supernatants was counted in a γ-counter. The percentage specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release) / (maximum release − minimum release).

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Three Immunoproteasome-Associated Subunits Cooperatively Generate a Cytotoxic T-Lymphocyte Epitope of Epstein-Barr Virus LMP2A by Overcoming Specific Structures Resistant to Epitope Liberation

Yoshinori Ito,[1] Eisei Kondo,[2] Ayako Demachi-Okamura,[1] Yoshiki Akatsuka,[1] Kunio Tsujimura,[1] Mitsune Tanimoto,[2] Yasuo Morishima,[3] Toshitada Takahashi,[1] and Kiyotaka Kuzushima[1]*

Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan[1]; Department of Internal Medicine II, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan[2]; and Department of Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan[3]

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The precise roles of gamma interferon-inducible immunoproteasome-associated molecules in generation of cytotoxic T-lymphocyte (CTL) epitopes have yet to be fully elucidated. We describe here a unique epitope derived from the Epstein-Barr virus (EBV) latent membrane protein 2A (LMP2A) presented by HLA-A*2402 molecules. Generation of the epitope, designated $LMP2A_{222–230}$, from the full-length protein requires the immunoproteasome subunit low-molecular-weight protein 7 (ip-LMP7) and the proteasome activator 28-α subunit and is accelerated by ip-LMP2, as revealed by gene expression experiments using an $LMP2A_{222–230}$-specific CTL clone as a responder in enzyme-linked immunospot assays. The unequivocal involvement of all three components was confirmed by RNA interference gene silencing. Interestingly, the $LMP2A_{222–230}$ epitope could be efficiently generated from incomplete EBV-LMP2A fragments that were produced by puromycin treatment or gene-engineered shortened EBV-LMP2A lacking some of its hydrophobic domains. In addition, epitope generation was increased by a single amino acid substitution from leucine to alanine immediately flanking the C terminus, this being predicted by a web-accessible program to increase the cleavage strength. Taken together, the data indicate that the generation of $LMP2A_{222–230}$ is influenced not only by extrinsic factors such as immunoproteasomes but also by intrinsic factors such as the length of the EBV-LMP2A protein and proteasomal cleavage strength at specific positions in the source antigen.

Cytotoxic T lymphocytes (CTLs) recognize short peptide products processed from target proteins and presented by major histocompatibility complex (MHC) class I molecules. The first step in protein processing in the cytosol is cleavage by proteasomes, proteolytic complexes playing a critical role in the antigen processing pathway. Resultant peptides are translocated by the transporters associated with antigen processing into the endoplasmic reticulum, where they assemble with newly synthesized MHC class I molecules for transportation to the cell surface (12, 28, 33). Proteasome catalytic activity is exerted by the 20S core proteasome, a cylindrical structure composed of four stacked rings. The outer two rings consist of seven different α subunits, and the inner rings consist of seven different β-type subunits. Enzymatic activity is mediated by three of the β subunits, designated β1 (Y/δ), β2 (Z/MC14), and β5 (X/MB1) (33). Exposure of cells to gamma interferon (IFN-γ) during immune responses alters the proteasome activity qualitatively and quantitatively with the induction of three newly synthesized immunoproteasome β subunits, low-molecular-weight protein 2 (ip-LMP2) or β1i, multicatalytic endopeptidase complex-like 1 (MECL-1) or β2i, and ip-LMP7 or β5i. These become incorporated interdependently and replace the three constitutive β subunits in newly assembled immunoproteasomes (14, 22, 24). The expression of ip-LMP7 and/or ip-LMP2 is known to alter the proteasomal cleavage specificity for virus- and tumor-associated antigens (15, 39). Furthermore, the incorporation of ip-LMP7 is sufficient to alter cleavage properties of proteasomes although the role of its catalytic site remains unclear (8, 36, 38, 40). The expression of ip-LMP2 alone or with ip-LMP7 is also reported to change cleavage specificity (1, 19, 23), and effects of the two subunits have been observed in each subunit's knockout mice (4, 7, 45).

Besides its effects on immunoproteasomes, IFN-γ up-regulates expression of the proteasome activator 28 (PA28), which consists of two different subunits, α and β, that form a heptameric ring that binds to proteasomes and is thought to increase their rate of cleavage (39). Regarding contributions to epitope liberation, effects of the α subunit have been observed (10, 41) but findings are limited regarding the β subunit (41). Elucidating differential effects of the three immunoproteasome subunits and two PA28 subunits is clearly important for a better understanding of generation of CTL epitopes.

Recently, defective ribosomal products (DRiPs) from newly synthesized proteins, which are rapidly ubiquitylated and degraded by proteasomes, were shown to be the main sources of antigenic peptides (29, 35, 48, 49); this suggests that antigen structures are critical for efficient processing by proteasomes. While almost every amino acid residue can serve as a cleavage site, there are certain preferences (25, 27, 44). Because the C terminus of the CTL epitope is precisely determined by the proteasome, cleavage strength at specific positions, such as that immediately flanking the C terminus of the epitope, really affects epitope generation (14, 34). In fact, mutation in the

* Corresponding author. Mailing address: Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. Phone: 81-52-764-2990. Fax: 81-52-764-2990. E-mail: kkuzushi@aichi-cc.jp.

flanking region of epitopes has been shown to impair the processing by proteasomes (2, 37). Thus, structural features play an important role in epitope liberation and could influence the working of the five immunoproteasome-associated subunits.

We have previously shown the generation of an HLA-A*2402-restriced CTL epitope in the Epstein-Barr virus (EBV) latent membrane protein 2A (EBV-LMP2A), amino acids 222 to 230 (referred to as $LMP2A_{222-230}$), to be dependent on IFN-γ exposure (18). Differential expression of ip-LMP2, MECL-1, ip-LMP7, PA28α, and PA28β in various combinations has allowed us to selectively address the role of each subunit in the processing of the epitope independently of other IFN-γ-inducible proteins, and we have established that the generation of $LMP2A_{222-230}$ is cooperatively controlled by interplay among ip-LMP2, ip-LMP7, and PA28α. Moreover, these observations were supported by the results of RNA interference experiments. We have now extended our studies to demonstrate that LMP2A structural factors influence epitope liberation in various target cells.

MATERIALS AND METHODS

CTL clones and epitopes. EBV-specific CTL lines and clones were established as described earlier (18). Briefly, EBV-specific T-cell lines were generated from peripheral blood mononuclear cells after stimulation with HLA-A*2402-transfected, TAP-negative T2-A24 cells (18) pulsed with each epitope peptide or autologous EBV-carrying lymphoblastoid cell lines (LCLs). After several rounds of stimulation, CTL clones were established by a limiting dilution method. A polyclonal CTL line that was specific to the epitope $LMP2A_{419-427}$ (TYGPVFMCL) (21) was designated $LMP2A_{419-427}$-CTL, and CTL clones that were specific to the epitope $LMP2A_{222-230}$ (IYVLVMLVL) (18) were designated $LMP2A_{222-230}$-CTL.

Cell lines. T2-A24 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 800 μg/ml of G418 (Invitrogen Corp., Carlsbad, CA). HLA-A*2402-positive LCLs and PT67 cells (BD Bioscience Clontech, Palo Alto, CA), retroviral packaging cell lines, were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml of kanamycin (referred to as LCL medium). HLA-A*2402-positive LCLs expressing short hairpin RNA (shRNA) were maintained in LCL medium in the presence of 0.8 μg/ml of puromycin. HEK293 T cells (referred to as 293T; American Type Culture Collection, Manassas, VA) and HLA-A*2402-positive dermal fibroblast cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Expression vectors. Plasmids expressing various lengths of EBV-LMP2A and EBNA3A, from full-length proteins to minimal epitopes, were constructed as described previously (16, 18). Full-length HLA-A*2402, ip-LMP2, ip-LMP7, MECL-1, PA28α, and PA28β were amplified by reverse transcriptase (RT)-PCR from HLA-A*2402-positive LCLs, cloned into the pcDNA3.1(+) vector (Invitrogen Corp.), and sequenced. A plasmid containing a mutant EBV-LMP2A gene with alanine substituted for leucine at position 231 was constructed by PCR-based mutagenesis as described previously (16). This single amino acid substitution was intended to increase the proteasome cleavage strength, as predicted with the Prediction Algorithm for Proteasomal Cleavages I program (PAProC version 1.0; http://www.paproc.de/) (17, 26).

Transduction of 293T cells. The plasmids encoding HLA-A*2402 and EBV-LMP2A and at least one of those expressing ip-LMP2, ip-LMP7, MECL-1, PA28α, or PA28β were transfected into 293T cells using TransIT-293 transfection reagents (Mirus, Madison, WI). Briefly, 3×10^4 cells were transfected with 100 ng of each plasmid and 0.2 μl TransIT reagent per 100 ng DNA in various combinations in 96-well plates. After 24 h, these cells were used as stimulators in the enzyme-linked immunospot (ELISPOT) assay.

shRNA interference retrovirus vectors. The following small interfering RNA targets were selected in this study: ip-LMP2, AAGUGAAGGAGGUCAGGUAUA; ip-LMP7, AGAUUAACCCUUACCUGCUTT; and PA28α, AAGCCAACUUGAGCAAUCUGA. shRNA constructs included a TTCAAGAGA-loop separating the sense and antisense sequences followed by a 5T termination signal. These constructs were synthesized as two cDNA oligonucleotides, annealed, and ligated between the BamHI and EcoRI sites of the RNAi-Ready pSIREN-RetroQ vector (BD Biosciences Clontech). In addition, oligonucleotides with sequences selected by the company (BD Biosciences Clontech) as a negative control for gene silencing were annealed and inserted into the same vector.

Retrovirus production and infection. PT67 cells were plated on six-well culture plates, and a 4-μg aliquot of each retrovirus vector plasmid was transfected with Lipofectamine 2000 (Invitrogen Corp), according to the manufacturer's instructions. After culture in the presence of 2.5 μg/ml of puromycin for 14 days, the cells were incubated in medium without puromycin for another 48 h. The culture supernatant was collected, and debris was removed by centrifugation at $1,000 \times g$ for 10 min. A total of 1×10^6 LCLs were suspended in 1 ml of the virus-containing culture supernatant in each well of a 12-well plate, and polybrene was added to a final concentration of 10 μg/ml. Plates were centrifuged at $1,000 \times g$ at 32°C for 1 h and incubated at 37°C in a humidified incubator. The LCLs were then cultured in medium containing puromycin for 14 days. Expression of ip-LMP2, ip-LMP7, or PA28α in these LCLs was analyzed by Western blotting and RT-PCR for gene silencing.

Western blotting. Western blotting was performed as described previously with slight modifications (42). Briefly, aliquots of 130 μg protein were applied to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked with phosphate-buffered saline containing 10% low-fat dry milk and 0.1% Tween 20 overnight at 4°C, and probed with rabbit polyclonal antibodies specific to ip-LMP2, ip-LMP7, and PA28α (Affinity, Mamhead, United Kingdom), followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed, San Francisco, CA). Proteins were visualized using an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, United Kingdom).

RT-PCR. Total RNA was extracted from LCLs and reverse transcription was performed in 20-μl reactions containing random hexamers and 1-μg aliquots. The specific primer sets used to detect ip-LMP2, ip-LMP7, and PA28α were as follows: ip-LMP2 forward, 5′-GGTGGTGAACCGAGTGTTTGA-3′; ip-LMP2 reverse, 5′-GCCAAAACAAGTGGAGGTTCC-3′; ip-LMP7 forward, 5′-GATTGCAGCAGTGGATTCTCG-3′; ip-LMP7 reverse, 5′-GACATGGTGCCAAGCAGGTAA-3′; PA28α forward, 5′-ACCAAGACAGAGAACCTGCTCG-3′; and PA28α reverse, 5′-GGCCTTCAGATTGCTCAAGTTG-3′.

ELISPOT assays. ELISPOT assays were performed as previously described (18). In brief, a MultiScreen-HA plate (Millipore) was coated with anti-human IFN-γ monoclonal antibody (Endogen, Rockford, IL) and used as the assay plate. The following stimulator cells in 100 μl of LCL medium were seeded into the wells: (i) 293T cells cotransfected with plasmids expressing HLA-A*2402 and those expressing various lengths of EBV-LMP2A (in some experiments, cells were treated with puromycin at 1 μg/ml for 30 min); (ii) 293T cells cotransfected with plasmids encoding HLA-A*2402 and EBV-LMP2A and at least one of those two expressing ip-LMP2, ip-LMP7, MECL-1, PA28α, or PA28b; and (iii) LCLs transduced by retrovirus vectors expressing shRNA for either ip-LMP2, ip-LMP7, or PA28a.

$LMP2A_{222-230}$-CTLs or $LMP2A_{419-427}$-CTLs in 100 μl medium were introduced into each well and incubated for 20 h. To visualize spots, anti-human IFN-γ polyclonal antibody (Endogen), horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed) and substrate were used. All assays were performed in duplicate.

RESULTS

$LMP2A_{222-230}$ is not presented on target cells expressing full-length EBV-LMP2A. The $LMP2A_{419-427}$-CTL responded to 293T cells pulsed with the epitope peptide and to those expressing full-length EBV-LMP2A cotransfected with HLA-A*2402. However, the $LMP2A_{222-230}$-CTL responded to 293T cells expressing the minimal epitope, but not full-length EBV-LMP2A, cotransfected with HLA-A*2402 (Fig. 1).

As we reported previously, IFN-γ-treated fibroblasts transduced with full-length EBV-LMP2A were recognized by $LMP2A_{222-230}$-CTL, showing $LMP2A_{222-230}$ to be an IFN-γ-dependent epitope. This suggested that IFN-γ-induced immunoproteasome and PA28 subunits generate $LMP2A_{222-230}$ in the target cells (18).

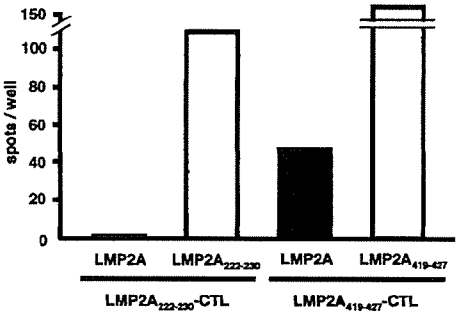

FIG. 1. EBV-specific CTL recognition of target cells measured by the ELISPOT assay. The epitope $LMP2A_{222-230}$ is not presented on 293T cells expressing full-length EBV-LMP2A while $LMP2A_{419-427}$ is presented on these cells. $LMP2A_{222-230}$-CTL is the clone specific for $LMP2A_{222-230}$, and $LMP2A_{419-427}$-CTL is a polyclonal $CD8^+$ T-cell line specific to $LMP2A_{419-427}$. 293T cells were cotransfected with plasmids expressing HLA-A*2402 and full-length EBV-LMP2A or pulsed with the epitope peptide. $CD8^+$ T cells (200/well) were cultured with the indicated stimulators for 20 h. Data from one representative experiment out of three are shown. Each bar demonstrates the average number of spots in duplicate wells.

Generation of $LMP2A_{222-230}$ requires the immunoproteasome subunit ip-LMP7 and PA28α and is enhanced by ip-LMP2. To investigate whether immunoproteasome-associated molecules are involved in generating the $LMP2A_{222-230}$ epitope, we examined the effect of each proteasome immunosubunit (ip-LMP2, ip-LMP7, and MECL-1) and PA28 subunit (PA28α and PA28β) in 293T cells that dominantly have a standard proteasome. First, 293T cells were cotransfected with plasmids encoding HLA-A*2402, the full-length EBV-LMP2A, and immunoproteasome-associated molecules in various combinations, as shown in Fig. 2A. We then evaluated epitope liberation using the ELISPOT assay. Surprisingly, three molecules were found to be involved in the generation of $LMP2A_{222-230}$: ip-LMP7 and PA28α subunits were required, and the ip-LMP2 subunit enhanced its recognition (Fig. 2A). We confirmed the expression of ip-LMP2, ip-LMP7, and PA28α by Western blotting (Fig. 2B).

Inhibition of ip-LMP2, ip-LMP7, and PA28α expression in LCLs by RNA interference decreases the generation of $LMP2A_{222-230}$ in target cells. LCLs predominantly have immunoproteasomes (24), and the $LMP2A_{222-230}$-CTL have recognized HLA-A*2402-positive LCLs, as we reported previously (18). To examine whether ip-LMP2, ip-LMP7, or PA28α is most directly involved in the generation of $LMP2A_{222-230}$, we evaluated the epitope liberation in LCLs in which the expression of each subunit was separately inhibited using a gene-silencing technique. HLA-A*2402-positive LCLs were infected with retrovirus vectors expressing shRNAs for ip-LMP2, ip-LMP7, or PA28α and assessed for the expression of each subunit by Western blotting (Fig. 3A, B, and C) and RT-PCR (data not shown). Then, generation of the $LMP2A_{222-230}$ epitope was probed with epitope-specific CTL using the ELISPOT assay. As expected, epitope liberation was clearly decreased with the inhibition of ip-LMP2, ip-LMP7, or PA28α expression (Fig. 3A, B, and C), demonstrating definitive involvement of all three molecules in the generation of $LMP2A_{222-230}$. To test whether the generation of IFN-γ-independent EBV-LMP2 epitope was influenced in these

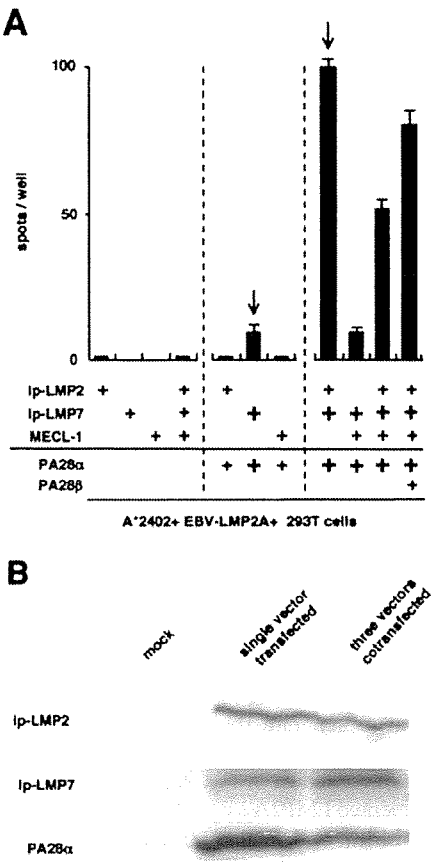

FIG. 2. Involvement of immunoproteasome and PA28 subunits in the generation of $LMP2A_{222-230}$ as analyzed by the ELISPOT assay. (A) Generation of $LMP2A_{222-230}$ requires three immunoproteasome-associated molecules. 293T cells were cotransfected with plasmids encoding HLA-A*2402, full-length EBV-LMP2A, and at least one immunoproteasome-associated molecule (ip-LMP2, ip-LMP7, MECL-1, PA28α, and PA28β). $LMP2A_{222-230}$-CTLs were cultured with stimulators for 20 h as described in Materials and Methods. Data from one representative experiment out of three are shown. Data are means plus or minus standard deviation (SD) of spots in duplicate wells. The arrows indicate noteworthy results. +, presence of immunoproteasome or subunit. (B) Expression of ip-LMP2, ip-LMP7, and PA28α in 293T cells transfected with corrresponding expression vectors. 293T cells were transfected with each of three plasmids or with all these vectors. Expression of each subunit was analyzed by Western blotting. "Single vector-transfected" represents the 293T cells transfected with each plasmid encoding ip-LMP2, ip-LMP7, or PA28α and "three vectors cotransfected" represents the 293T cells cotransfected with the three plasmids. Results of one representative experiment out of two are shown.

LCLs transfected with shRNA expression vectors for ip-LMP2, ip-LMP7, or PA28α, we investigated the generation of $LMP2A_{419-427}$ using the ELISPOT assay. We found that there were no significant differences in the processing of this epitope. (data not shown).

Incomplete or shortened EBV-LMP2A results in efficient generation of $LMP2A_{222-230}$ in target cells. Recently, DRiPs have been reported to be major sources of CTL epitopes (43, 46), suggesting that incomplete antigen proteins allow efficient processing. To test this possibility with regard to $LMP2A_{222-230}$, 293T

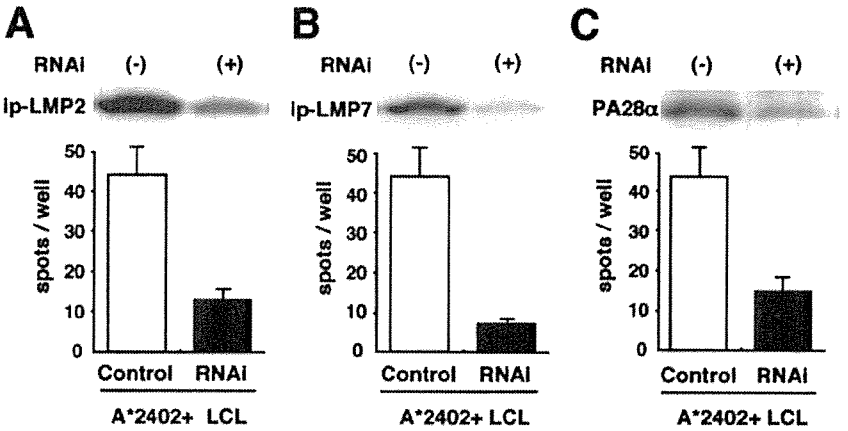

FIG. 3. Generation of $LMP2A_{222-230}$ is inhibited by RNA interference (RNAi) products targeting ip-LMP2 (A), ip-LMP7 (B), or PA28α (C) expression. Immunoproteasome-expressing HLA-A*2402-positive LCLs were infected with retrovirus vectors expressing shRNA for ip-LMP2, ip-LMP7, or PA28α. As a control stimulator, an LCL infected with a retrovirus vector expressing a nonsilencing shRNA was used. Inhibition of each subunit expression in shRNA-expressing LCLs was analyzed by Western blotting. $LMP2A_{222-230}$-CTL (5×10^3 cells/well) was cultured with each shRNA-transduced LCL for 20 h. Results of one representative experiment out of two are shown. Data are means plus or minus SD of spots in duplicate wells. −, absence of immunoproteasome or subunit; +, presence of immunoproteasome or subunit.

cells transduced with HLA-A*2402 and full-length EBV-LMP2A together with ip-LMP7 and/or PA28α were treated with puromycin for 30 min to generate short-lived premature proteins (6, 13, 47). We then analyzed the generation of $LMP2A_{222-230}$ by ELISPOT assay. As shown in Fig. 4A, puromycin treatment remarkably augmented $LMP2A_{222-230}$-CTL recognition on the cells expressing ip-LMP7 and PA28α. Interestingly, puromycin was capable of substituting either effect of ip-LMP7 and PA28α.

Next, we introduced truncated EBV-LMP2A of different lengths starting from isoleucine at position 222, the first amino acid of $LMP2A_{222-230}$, into expression vectors (Fig. 4B). The generation of $LMP2A_{222-230}$ was studied in 293T cells cotransfected with vectors encoding HLA-A*2402 and each truncated EBV-LMP2A without immunoproteasomes and PA28 subunits. Interestingly, the shortest EBV-LMP2A antigen was processed most efficiently and all truncated EBV-LMP2A antigens could be processed to generate $LMP2A_{222-230}$ without the aid of immunoproteasomes and PA28 (Fig. 4C). These data clearly demonstrated that the efficiency of $LMP2A_{222-230}$ generation is, at least in part, dependent on the length of the source protein.

Substitution of amino acid immediately flanking the C terminus of $LMP2A_{222-230}$, increasing the proteasome cleavage strength, results in efficient generation of $LMP2A_{222-230}$ in target cells. Finally, we investigated whether the amino acid cleavage strength at a specific position affects the processing of the $LMP2A_{222-230}$ epitope. To determine the cleavage strength of each amino acid in EBV-LMP2A, the program PAProC was used (http://www.paproc.de/). We focused on the cleavage strength, which is critical for epitope generation, of amino acids in the position immediately flanking the C termini of CTL epitopes (14, 34). First, we constructed a plasmid containing a mutant full-length LMP2A gene in which alanine replaced leucine at position 231; this was predicted to increase the cleavage strength after leucine at position 230, i.e., the C terminus of $LMP2A_{222-230}$ (Fig. 5A). It was thought that this change would facilitate $LMP2A_{222-230}$ generation by proteasomes. Target 293T cells were cotransfected with vectors encoding HLA-A*2402, ip-LMP7, PA28α, and the mutant EBV-LMP2A, and $LMP2A_{222-230}$-CTL recognition was evaluated using the ELISPOT assay. A remarkable increase was evident for cells expressing ip-LMP7 and PA28α (Fig. 5B), suggesting the processing of $LMP2A_{222-230}$ to be accelerated by the amino acid substitution at the specific position in the EBV-LMP2A antigen.

DISCUSSION

IFN-γ induces cells to express the proteasome subunits ip-LMP2, MECL-1, and ip-LMP7, leading to the formation of immunoproteasomes and the proteasome activator subunits PA28α and PA28β, comprising the activator complex. Early experiments with IFN-γ-treated cells demonstrated the generation of a number of epitopes to be affected by immunoproteasomes and PA28 (15, 32, 44). Immunoproteasomes have various cleavage site preferences as well as cleavage rates for the generation of some epitopes, while PA28 up-regulates epitope liberation via conformational changes within the proteasome 20S complex. Following the discovery that the influenza virus matrix-derived epitope required ip-LMP7 expression for its generation (3), the involvement of immunoproteasomes and PA28 subunits with different CTL epitopes received much attention. The results of the studies that investigated the effect of at least two immunoproteasome-associated molecules in the generation of CTL epitopes are summarized in Table 1 (1, 8, 10, 19, 23, 36, 38, 40, 41). The combination patterns of the five immunoproteasome-associated subunits fall into three categories. (i) PA28 alone, (ii) ip-LMP7 alone, and (iii) both ip-LMP2 and ip-LMP7 exerted the epitope generation. It has been hypothesized that immunoproteasomes and PA28 cooperate in antigen processing, but direct experimental evidence has hitherto been lacking. In this study, we found that the $LMP2A_{222-230}$ epitope has two unique features. First, coexpression of ip-LMP7 and one PA28 subunit is necessary for its

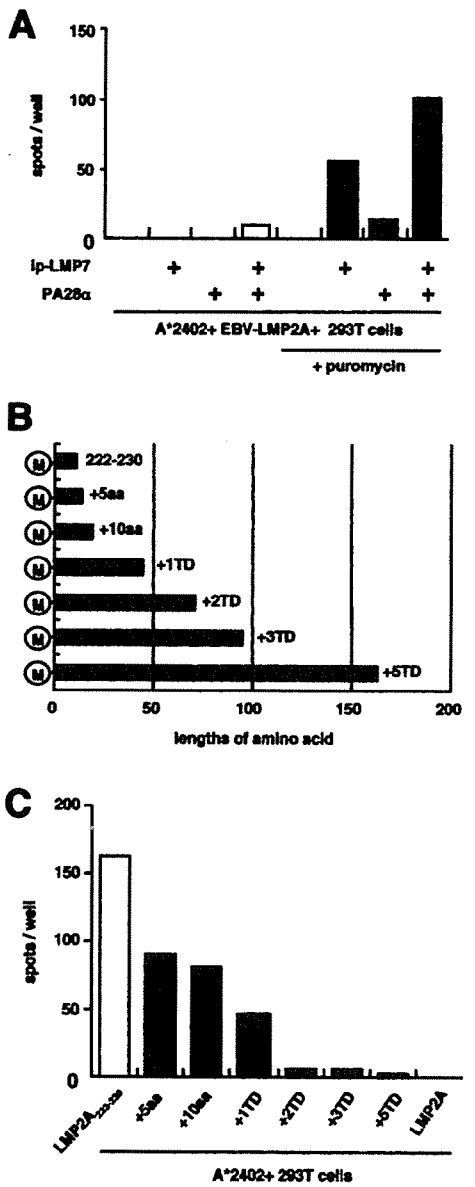

FIG. 4. Generation of $LMP2A_{222-230}$ from incomplete and shortened EBV-LMP2A as analyzed by ELISPOT assay. (A) $LMP2A_{222-230}$-CTL recognition of puromycin-treated (1 μg/ml for 30 min) or untreated target cells expressing EBV-LMP2A. 293T cells, cotransfected with plasmids encoding HLA-A*2402, full-length EBV-LMP2A, ip-LMP7, and/or PA28, were cultured with $LMP2A_{222-230}$-CTL (1×10^4 cells/well) for 20 h. For Ip-LMP7 and PA28 lanes, "+" indicates the presence of the immunoproteasome or subunit. (B) The length and structure of the truncated EBV-LMP2A fragments. Numbers of transmembrane domains (TDs) following $LMP2A_{222-230}$ in the fragments are shown; e.g., +3TD indicates fragment $LMP2A_{222-230}$ plus three transmembrane domains. Each TD located from the C terminus of $LMP2A_{222-230}$ is serially numbered. M, methionine. (C) $LMP2A_{222-230}$-CTL recognition of target cells expressing truncated EBV-LMP2A fragments in the absence of immunoproteasomes. The 293T cells were cotransfected with expression vectors encoding HLA-A*2402 and one truncated EBV-LMP2A and cultured with $LMP2A_{222-230}$-CTL (1×10^3 cells/well) for 20 h. Numbers of amino acids and transmembrane domains (TDs) following $LMP2A_{222-230}$ in the fragments are shown; e.g., +5aa indicates fragment $LMP2A_{222-230}$ plus five C-terminal amino acids. Results of one representative experiment out of two are shown. Each bar represents the average number of spots in duplicate.

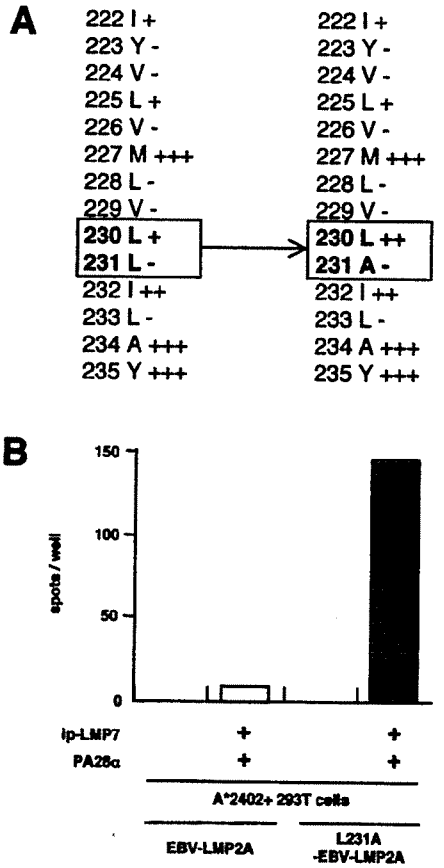

FIG. 5. Comparison of generation of $LMP2A_{222-230}$ from native and mutant EBV-LMP2A with alanine substituted for leucine at position 231 (referred as to L231A-EBV-LMP2A). (A) Partial amino acid sequences of EBV-LMP2A and L231A-EBV-LMP2A. The position numbers, single code letters for amino acids and predicted cleavage strengths are shown. Predictions by the program PAProC are scored as follows: −, no cleavage behind this position; +, ++, +++, cleavage behind this position, with a hint of the strength indicated by the number of +'s. (B) Generation of $LMP2A_{222-230}$ from EBV-LMP2A and L231A-EBV-LMP2A, analyzed by ELISPOT assay. 293T cells were cotransfected with plasmids encoding HLA A*2402, ip-LMP7, PA28α, and full-length EBV-LMP2A or L231A-EBV-LMP2A and cultured with $LMP2A_{222-230}$-CTL for 20 h. Results of one representative experiment out of two are shown. Each bar represents the average number of spots in duplicate. For Ip-LMP7 and PA28 lanes, "+" indicates the presence of the immunoproteasome or subunit.

generation. Second, ip-LMP2 has additional effects on epitope liberation. These data suggest that the processing of an IFN-γ-inducible epitope is controlled differentially by multiple immunoproteasome-associated subunits. To our best knowledge, this is the first documentation of molecular evidence of such cooperation.

Incorporation of the immunoproteasome is reported to be cooperative. The ip-LMP7 is required for immunoproteasome formation and maturation (9, 14). MECL-1 is incorporated if ip-LMP2 is present, while MECL-1 dependency for the incorporation of ip-LMP2 is under dispute (5, 11). Moreover, this cooperativity in forming proteasome complexes results in altered cleavage properties. In the present study, the generation

TABLE 1. Effects of immunoproteasomes and PA28 subunits by species on epitope generation[a]

Species	Source	Antigen	Epitope location	Epitope sequence	Immunoproteasome or subunit					Reference
					ip-LMP2	MECL1	ip-LMP7	PA28α	PA28β	
Mouse	Murine CMV	pp89	168–176	YPHFMPTNL	−	ND[b]	−	+	ND	11
Mouse	Influenza virus	NP	146–154	TYGRTRALV	−	ND	−	+	ND	11
Human	Influenza virus	Matrix	58–66	GILGFVFTL	−	ND	+	ND	ND	9
Human	HIV	RT	346–354	VIYQYMDDL	−	ND	+	ND	ND	38
Human	HBV	HBcAg	141–151	STLPETTVVRR	−	−	+	ND	ND	39
Human	Melanoma	MAGE-3	114–122	AELVHFLLL	−	−	+	ND	ND	36
Human	Melanoma	TRP2	360–368	TLDSQVMSL	−	−	−	+	+	41
Human	EBV	LMP2A	356–364	FLYALALLL	+	−	+	−	−	20
Human	Melanoma	Melan-A	26–35	ELAGIGILTV	+	−	+	ND	ND	23
Human	LCMV	gp	33–41	KAVYNFATC	+	ND	+	−	−	1
Human	LCMV	gp	276–286	SGVENPGGYCL[c]	+	ND	+	−	−	1
Human	EBV	LMP2A	222–230	IYVLVMLVL	+	−	+	+	−	Present study

[a] CMV, cytomegalovirus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; LCMV, lymphocytic choriomeningitis virus; −, absence; +, presence.
[b] ND, not done.
[c] Generation of this epitope was inhibited by the expression of either ip-LMP7 or ip-LMP2.

of $LMP2A_{222-230}$ is enhanced by ip-LMP2 expression. This effect may be exerted through the functions of ip-LMP7 and PA28α, which induce the cleavages properties on the epitope generation.

In this study, we developed a retrovirus vector producing shRNA to confirm the effects of ip-LMP2, ip-LMP7, and PA28α in the generation of $LMP2A_{222-230}$. Generally, the use of chemically synthesized small interfering RNA or expression plasmids for shRNA is a more feasible way to test the involvement of target molecules but we believe that the retrovirus system has advantages in our case, because the effects of RNA interference in the target cells proved stable. After an epitope binds to MHC molecules and is presented on the cell surface, the complex exists for some time. Since there is a wide range in the life spans of the MHC-epitope complex, it is difficult to infer the sufficient duration to maintain inhibition of immunoproteasome-associated subunits to examine their effects in peptide liberation. In our retrovirus system, LCLs were cultured in medium containing puromycin for 14 days after retrovirus vector infection, and we assessed $LMP2A_{222-230}$ presentation on the surface. This procedure should exclude false-positive results that are observed with the ELISPOT assay.

DRiPs are thought to be important sources of CTL epitopes (29, 35, 49), as in the case of EBNA1, for example, for which epitopes are not readily generated from stable mature EBNA1 because of the glycine-alanine repeat domain within the protein (43, 46). EBV-LMP2A has 12 hydrophobic integral membrane sequences, and this hydrophobic-rich structure may inhibit epitope liberation (20). To address the question of whether the incomplete EBV-LMP2A might be superior to the mature complete EBV-LMP2A for epitope generation, we treated target cells with puromycin, which generates short-lived premature termination products from newly synthesized proteins (6, 13, 47). Interestingly, $LMP2A_{222-230}$ production was accelerated in puromycin-treated 293T cells expressing ip-LMP7 or PA28α, in contrast to the limited yield without puromycin treatment, even when the subunits were coexpressed. The data suggest that puromycin treatment is not sufficient to generate $LMP2A_{222-230}$ epitopes via constitutive proteasomes, but rather affects epitope generation by enhancing the effect of ip-LMP7 and PA28α. Next, we expressed a panel of shorter EBV-LMP2A fragments encompassing $LMP2A_{222-230}$ in target cells and compared their recognition to that of $LMP2A_{222-230}$-CTL. Each fragment started from the N terminus of $LMP2A_{222-230}$, as shown in Fig. 4B. This strategy should focus on the cleavage efficiency of the C-terminal side, which is performed exclusively by proteasomes (14, 34). We found that shorter EBV-LMP2A fragments were processed more efficiently. Therefore, the length of the source antigen may be a critical factor. Addition of two consecutive hydrophobic transmembrane domains substantially abrogated the epitope presentation. The obstacles presented by the intrinsic structure of EBV-LMP2A may be overcome by the effects of ip-LMP7 and PA28α in the generation of the $LMP2A_{222-230}$ epitope.

The cleavage efficiency at each amino acid varies widely in antigen proteins (25, 27, 44), and this may explain why one epitope is generated efficiently by proteasomes while another is not, even when processed from the same protein. Previous work showed that even a single amino acid substitution of asparagine for the aspartic acid immediately flanking the C terminus of the Moloney murine leukemia virus epitope SSWDFITV resulted in its abrogation (2). The program PAProC predicts that the cleavage strength of the C-terminal leucine in EBV-LMP2A is weak (17, 26), and substitution of an amino acid to increase the cleavage strength (from "+" to "++", as shown in Fig. 5A) resulted in remarkable up-regulation of $LMP2A_{222-230}$ liberation in cells expressing ip-LMP7 and PA28α.

EBV-LMP2A is thought to be an important antigen in EBV-related malignancies and is targeted by CTLs that recognize multiple epitopes located throughout the membrane-spanning molecules (20, 30, 31). Interestingly, EBV-LMP2A epitopes can be divided into two groups: (i) hydrophobic examples located in the transmembrane domain and processed in a TAP-independent manner and (ii) intertransmembrane hydrophilic epitopes, which are TAP-dependent (20). In addition, the generation of one hydrophobic epitope, $LMP2A_{356-364}$, requires ip-LMP7 and ip-LMP2 (19). Moreover, we here demonstrated that the processing of $LMP2A_{222-230}$ requires immunoproteasome subunits ip-LMP7 and PA28α and is enhanced by immunoproteasome subunit ip-LMP2. These two epitopes belong to

the former group, although the effects of immunoproteasome and PA28 subunits on other epitopes remain to be investigated. Potentially, EBV-LMP2A is a good model for determining the mechanisms by which immunoproteasomes and PA28 affect CTL epitope generation.

In conclusion, the present investigation provided evidence for differential roles of ip-LMP2, ip-LMP7, and PA28α in the generation of the $LMP2A_{222-230}$ epitope, which was most efficiently generated from incomplete EBV-LMP2A fragments and a mutated LMP2A gene with improved cleavage characteristics in cells expressing ip-LMP7 and PA28α. Although the precise function of each of the three subunits could not be clarified, we showed the generation of $LMP2A_{222-230}$ to be controlled by multiple factors. Further investigations on the differential effects of immunoproteasome-associated subunits could provide important information for understanding the presentation of viral and tumor antigens for CTL recognition.

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Bone marrow may be a reservoir of long-lived memory T cells specific for minor histocompatibility antigen

It has been shown that minor histocompatibility antigens (mHAgs) can function as targets for the graft-*versus*-leukaemia effect (Goulmy, 1997) following human leucocyte antigen (HLA)-identical allogeneic haematopoietic cell transplantation (HCT) and donor lymphocyte infusion (Marijt *et al*, 2003). We previously identified two haematopoietic-specific mHAgs, ACC-1 and ACC-2 (Akatsuka *et al*, 2003), and demonstrated that T cells specific for ACC-1 were detected in the peripheral blood (PB) up to 7 month postHCT in a patient from whom the original ACC-1-specific cytotoxic T cell (CTL) clone had been generated (Nishida *et al*, 2004). As it has recently been proposed that bone marrow (BM) can function as a secondary lymphoid organ and contribute to long-term T cell memory for pathogens and malignant disease (reviewed in Di Rosa & Pabst, 2005), this study was conducted to investigate whether T cells specific for mHAgs could feasibly be generated from BM, rather than PB, long after HCT.

Another patient who received an HLA-identical, ACC-1-disparate HCT for chronic myelomonocytic leukaemia was identified. At 14 months postHCT, she had complete donor chimerism in PB and remained disease free. After informed consent, we examined the phenotype and proliferative capacity of ACC-1-specific T cells in mononuclear cells (MCs) obtained from the patients' PB and BM. Three-colour flow cytometry detected a 3·5-fold higher percentage of ACC-1-specific T cells among the $CD8^+$ population in BM than PB (0·72% vs. 0·21%), with a trend of more $CD62L^+$ cells in the BM (Fig 1A, upper panels). After $CD8^+$ cell selection by immunomagnetic beads, $tetramer^+$ cells were further stained with antibodies

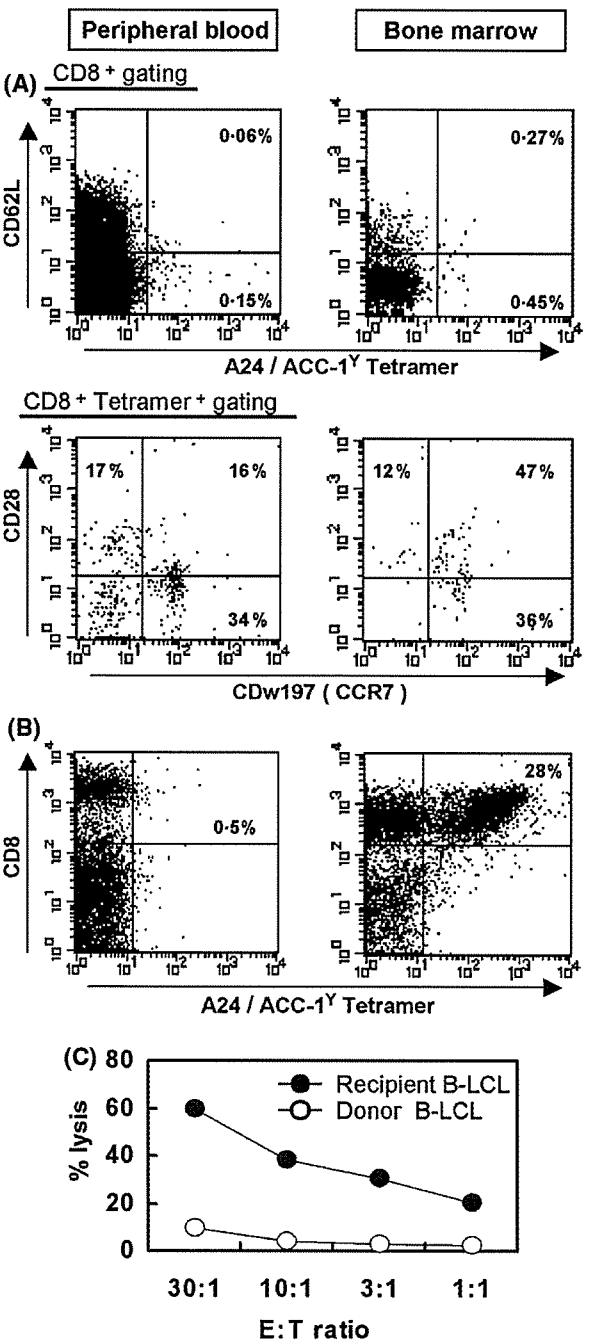

Fig 1. Characterisation of $CD8^+$ T cells specific for the ACC-1 minor histocompatibility antigen in peripheral blood (PB) and bone marrow (BM) at 14 months following human leucocyte antigen (HLA)-identical allogeneic haematopoietic cell transplantation. (A) Mononuclear cells (MC) were isolated from PB and BM and stained with fluorescence-conjugated monoclonal antibodies and HLA-A24/ACC-1 tetramer (Nishida *et al*, 2004). In lower panels, $CD8^+$ MC were first sorted and then stained as above. Percentages shown are of the gated T cells as indicated. (B) Remaining MC were stimulated with 0·1 μmol/l ACC-1 peptide (DYLQYVLQI) directly added to cell suspension on day 0 and 7 in RPMI 1640 medium supplemented with 6% pooled human serum. On day 14, donor-derived OKT3-activated $CD4^+$ blasts, pulsed with the same concentration of peptide, were added as antigen-presenting cells. Interleukin-2 (10 U/ml) was added on days 1 and 4 after the second and third stimulations. Growing T cells were stained as above. (C) Cytolytic activity of the T cell line generated from BM in the right panel of Fig 1B is shown.

against CD28, CDw197 (CCR7), CD44 (H-CAM, as a marker for memory T cells) and CD49d (VLA4α, a receptor for vascular cellular adhesion molecule-1 expressed on BM stromal cells). BM tetramer$^+$ cells contained a higher percentage of CCR7$^+$ cells than PB (83% vs. 50%), and a threefold higher percentage of CD28$^+$ cells were detected in the BM tetramer$^+$ CCR7$^+$ population (47% vs. 16%, Fig 1A lower panel). More than 97% of the cells were CD44$^+$ and CD49d$^+$ in both PB and BM (data not shown). These findings indicate that the vast majority of tetramer$^+$ cells were memory T cells that possessed the property of being able to migrate to BM, and that most tetramer$^+$ cells in BM also expressed CCR7, a lymph node homing receptor, as a central memory T (T_{CM}) cell marker. It is of note that more than half of BM ACC-1-specific CTL expressed CD28, an important costimulatory molecule for effective CD8$^+$ T cell responses that interacts with CD80/86 expressed by antigen-presenting cells to stimulate interleukin-2 production (Topp *et al*, 2003).

Next we tested whether these tetramer$^+$ cells could be expanded by ACC-1 peptide stimulation. Peripheral blood mononuclear cells (PBMC) or bone marrow mononuclear cells (BMMC) from the same aliquot ($c. \times 10^6$) were stimulated twice in the presence of 0·1 μmol/l (predetermined concentration) of ACC-1 peptide alone, and once with ACC-1-pulsed (0·1 μmol/l), donor-derived antigen-presenting cells. Staining with tetramer was done 21 d after the first stimulation. As shown in Fig 1B, stimulated BMMC produced a large population of tetramer$^+$ cells, whereas PBMC did not yield any significant tetramer$^+$ population (confirmed in an independent experiment, data not shown). The T cell line induced from the BMMC was cytotoxic to recipient B-lymphoblastoid cells (Fig 1C). The reason for the poor induction of tetramer$^+$ cells from the PBMC at 14 month postHCT is most probably a result of their reduced capacity to proliferate *in vitro* long after HCT, as PBMC harvested on days 94 and 180 postHCT readily generated 37% and 18% of tetramer$^+$ populations respectively (data not shown). These observations suggest that BM may be a superior source of mHAg-specific T_{CM} that could possibly be expanded and used for immunotherapy, although it is to be determined whether our finding is a general phenomenon not only in other HCT cases, but also in other mHAgs.

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Yoshiki Akatsuka[1]
Hiroki Torikai[1]
Yoshihiro Inamoto[2]
Kunio Tsujimura[1]
Yasuo Morishima[3]
Yoshihisa Kodera[2]
Kiyotaka Kuzushima[1]
Toshitada Takahashi[1]

[1]*Division of Immunology, Aichi Cancer Centre Research Institute, Nagoya,* [2]*Department of Haematology, Japanese Red Cross Nagoya First Hospital, Nagoya, and* [3]*Department of Haematology and Cell Therapy, Aichi Cancer Centre Hospital, Nagoya, Japan.*
E-mail: yakatsuk@aichi-cc.jp

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bjh research paper

The human *cathepsin H* gene encodes two novel minor histocompatibility antigen epitopes restricted by HLA-A*3101 and -A*3303

H. Torikai,[1,2] Y. Akatsuka,[1] M. Miyazaki,[1] A. Tsujimura,[3] Y. Yatabe,[4] T. Kawase,[1] Y. Nakao,[1] K. Tsujimura,[1] K. Motoyoshi,[2] Y. Morishima,[3] Y. Kodera,[5] K. Kuzushima[1] and T. Takahashi[1]

[1]*Division of Immunology, Aichi Cancer Centre Research Institute, Nagoya,* [2]*Third Department of Internal Medicine, National Defence Medical College, Tokorozawa,* [3]*Department of Haematology and Cell Therapy, Aichi Cancer Centre Central Hospital,* [4]*Department of Pathology and Molecular Diagnostics, Aichi Cancer Centre Central Hospital, and* [5]*Department of Haematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan*

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Correspondence: Dr Y Akatsuka, Division of Immunology, Aichi Cancer Centre Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. E-mail: yakatsuk@aichi-cc.jp

Summary

Minor histocompatibility antigens (mHags) play crucial roles in the induction of graft *versus* host disease (GVHD) and/or graft *versus* leukaemia (GVL) effects following human leucocyte antigen (HLA)-identical haematopoietic stem cell transplantation (HSCT). Using HLA-A*3101- and -A*3303-restricted cytotoxic T lymphocyte (CTL) clones generated from different post-HSCT recipients, we identified two novel mHag epitopes encoded by the leader sequence of cathepsin H (CTSH) isoform a. The nonameric sequence ATLPLLCAR was defined as an HLA-A*3101-restricted epitope ($CTSH^R$/A31), while a decameric peptide featuring a one N-terminal amino acid extension, WATLPLLCAR, was presented by HLA-A*3303 ($CTSH^R$/A33). The immunogenicity of both epitopes was totally dependent on the polymorphic C-terminal arginine residue and substitution with glycine completely abolished binding to the corresponding HLA molecules. Thus, the immunogenicity of this mHag is exerted by differential HLA binding capacity. CTSH is relatively ubiquitously expressed at protein levels, thus it may be involved in GVHD and anti-leukaemic/tumour responses. Interestingly, however, CTL clones predominantly lysed targets of haematopoietic cell origin, which could not be explained in terms of the immunoproteasome system. Although the mechanisms involved in the differential susceptibility remain to be determined, these data suggest that CTSH-encoded mHags could be targets for GVL effects.

Keywords: minor histocompatibility antigen, graft *versus* leukaemia, graft *versus* host disease, cytotoxic T lymphocyte, cathepsin H.

Minor histocompatibility antigens (mHags) are major histocompatibility complex (MHC)-bound peptides derived from cellular proteins that are encoded by polymorphic genes, mostly due to single nucleotide polymorphism (SNP) (Goulmy *et al*, 1996; Simpson & Roopenian, 1997). Disparities in some mHags with allogeneic haematopoietic stem cell transplantation (HSCT) have been shown to be associated with graft *versus* host disease (GVHD) (Goulmy *et al*, 1996; Tseng *et al*, 1999; Akatsuka *et al*, 2003a), graft rejection (Marijt *et al*, 1995) and/or graft *versus* leukaemia (GVL) effects (Bonnet *et al*, 1999; Marijt *et al*, 2003). Although methods for identification of MHC-bound antigens have improved, only a limited number of mHags have been reported to date (Bleakley & Riddell, 2004; Spierings *et al*, 2004). Thus, identification of novel mHags should facilitate further understanding of the mechanisms involved in generating mHags and also of the pathogenesis of GVHD, which may lead to development of effective immunotherapy.

This study identified the mHag epitopes recognised by two HLA-A*3101 and -A*3303-restricted cytotoxic T lymphocyte (CTL) clones, which were encoded by the same non-synonymous SNP in the *cathepsin H* (*CTSH*) gene. The polymorphic amino acid (aa) responsible for antigenicity was an arginine (Arg) in place of glycine (Gly) at the C terminus of the identified epitopes. The latter substitution resulted in more profound loss of peptide-HLA binding than previously demonstrated with HA-1/A2 (den Haan *et al*, 1998) and HA-2 (Pierce *et al*, 2001). CTSH has been shown to be expressed relatively

ubiquitously, but unexpectedly, CTL clones specific for CTSH-encoded mHag lysed haematopoietic cells, including primary acute myeloid leukaemia (AML) cells obtained from patients, but not some cytokine-treated non-haematopoietic cells, even though high levels of CTSH protein expression and immunoproteasome components comparable with those of haematopoietic cells were found. The selective susceptibility of haematopoietic cells suggests that CTSH should be further evaluated as a potential target to augment GVL effects.

Materials and methods

Patients, cell collection and cultures

Cytotoxic T lymphocyte clones, clinical characteristics of the two patients from whom the clones were obtained with unique patient numbers (UPNs) 027 and 028, are summarised in Table I. This study was approved by the Institutional Review Board of the Aichi Cancer Centre according to the Declaration of Helsinki. All blood or tissue samples were collected after written informed consent was obtained. Epstein–Barr virus-transformed B lymphocyte cell lines (B-LCL) were derived from donors, recipients, and normal volunteers and maintained in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (IBL, Takasaki, Japan). Individual patients' primary fibroblast lines established pre-HSCT from skin and bone marrow, normal renal epithelial cells and renal cell carcinoma cell lines established from restriction HLA-matched unrelated individuals and 293T cells were grown in Iscove's-modified Dulbecco's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum. B-LCLs from Centre d'Etude du Polymorphism Humain (CEPH) families (provided by Dr P. Martin, Human Immunogenetics Program, Fred Hutchinson Cancer Research Center, Seattle, WA, USA) were transduced with HLA-A*3101 or -A*3303 cDNA-encoding retroviral vectors as described previously (Akatsuka *et al*, 2003b).

Generation and cytotoxicity analysis of CTL lines and clones

Cytotoxic T lymphocyte lines were generated from post-HSCT peripheral blood mononuclear cells (PBMC) (*c.* 1×10^6) by primary stimulation with irradiated (33 Gy) pre-HSCT recipient PBMC (*c.* 1×10^6) followed by weekly restimulation with irradiated (33 Gy) recipient activated B cells (2×10^6, see below) twice. Interleukin (IL)-2 (20 U/ml; Chiron, Emeryville, CA, USA) was added on days 1 and 5 after the second and third stimulation. CTL clones were generated by limiting dilution as previously reported (Akatsuka *et al*, 2002). The CTLs were expanded as previously described (Walter *et al*, 1995) and frozen until use. All cultures were performed in RPMI 1640 medium supplemented with 9% pooled human serum. The cytotoxic activity of the CTL lines and clones was evaluated by standard ^{51}Cr release assays. Some target cells were treated with cytokines as indicated for 48 h. Percentage-specific lysis was calculated as follows: ((experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)) × 100.

For antibody blocking experiments, cells were incubated for 30 min with predetermined concentrations of monoclonal antibodies W6/32 (anti-HLA class I) or HDR-1 (anti-HLA-DR; provided by K. Ito, Kurume University, Fukuoka, Japan) before mixing with CTL clones. The antigen specificity of tumour cell lysis was further determined in a cold target inhibition assay by adding unlabelled cold target cells (indicated) to labelled target cells (hot targets) at serial ratios of cold-to-hot target cells. The percentage of inhibition was calculated as follows: ((% specific lysis without cold target − % specific lysis with cold target)/(% specific lysis without cold target)) × 100.

Table I. Characteristics of patients and cytotoxic T lymphocyte clones.

	UPN 027	UPN 028
Disease	AML with multilineage dysplasia	AML (FAB classification, M5b)
Status at HSCT	Resistant disease	First relapse
Donor	Matched sibling (male to male)	Matched sibling (male to male)
HLA	A24/33, B44/75, Cw3/−, DR4/6	A31/33, B44/51, Cw3/−, DR4/13
Acute GVHD	Grade I (skin stage 2)	None
Chronic GVHD	None	Extensive but mild (oral, skin)
Other complications	BOOP, IgG deposition at glomerulus	None
Current status	Alive disease-free over 3 years	Alive disease-free over 2·5 years
Representative CTL	Clone 2A10	Clone 1A8
Days obtained	31 d post-HSCT	29 d post-HSCT
HLA restriction	HLA-A*3303	HLA-A*3101

UPN, unique patient number; AML, acute myeloid leukaemia; FAB, French–American–British classification; HSCT, haematopoietic stem cell transplantation; HLA, human leucocyte antigen; GVHD, graft *versus* host disease; BOOP, bronchiolitis oblitarans organising pneumonia; CTL, cytotoxic T lymphocyte; Ig, immunoglobulin.

Construction of a cDNA library and expression screening

A cDNA library was constructed using the SuperScript Plasmid System (Invitrogen, Carlsbad, CA, USA). A sample (2 μg) of messenger RNA isolated from B-LCL recognised by CTL clones with a FastTrack 2·0 kit (Invitrogen) was converted into cDNA using an oligo-dT primer containing a NotI site in its 3′-end and SuperScript II reverse transcriptase (Invitrogen). cDNA was ligated to SalI adaptors, and then digested with NotI, size fractionated by column chromatography and ligated into the SalI and NotI cut pCMVSPORT6 vector (Invitrogen). Recombinant plasmids were transformed into *Escherichia coli* DH10B by electroporation, and clones were selected with ampicillin. The library contained $1{\cdot}5 \times 10^6$ cDNA clones with an average insert size of approximately 2500 bp. cDNA pools, each consisting of *c.* 120 clones were expanded for 24 h in 96-deep well plates, and plasmid DNA was extracted with a QIAprep 96 Turbo Miniprep kit (Qiagen, Valencia, CA, USA).

The 293T cells ($n = 20\,000$), stably transduced with HLA-A*3303 or -A*3101 were plated in 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0·12 μg of plasmid containing a pool of the cDNA library using Trans IT-293 (Mirus Technologies, Madison, WI, USA). CTL clones (10 000 cells/well) were added to each well 20 h after transfection. After overnight incubation in the presence of 10 U/ml IL-2 at 37°C, 50 μl of supernatant was collected and interferon (IFN)-γ was measured by enzyme-linked immunosorbent assay (ELISA) with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich).

Genotyping of CTSH polymorphisms

Genomic DNA was isolated from each B-LCL with a DNA blood kit (Qiagen) and amplified by polymerase chain reaction (PCR). The primer sequences used to amplify *CTSH* exon 1 were as follows: sense, 5′-GAACTAGAGCTGGGGAGTTA-3′ antisense, 5′-CCCGCCTATAATGCAGTTTA-3′. PCR products were purified and directly sequenced with the same primer and a BigDye Terminator kit (ver. 3·1; Applied Biosystems, Foster City, CA, USA) using an ABI PRISM 3100 (Applied Biosystems).

HLA peptide-binding assay

We used a quantitative ELISA-based assay capable of measuring the affinity of the interaction between peptide and HLA as described previously, with some modifications (Sylvester-Hvid *et al*, 2002). In brief, purified recombinant HLA molecules in 8 mol/l of urea, 10 mmol/l of EDTA, 25 mmol/l of 2-(*N*-morpholino)ethanesulfonic acid (MES) and 0·1 mmol/l of dithiothreitol were diluted to 4 μg/ml in refolding buffer containing 400 mmol/l of Arg, 100 mmol/l Tris pH 8·0, 2 mmol/l of EDTA, 5 mmol/l of reduced glutathione, 0·5 mmol/l of oxidised glutathione, 0·2 mmol/l of phenyl methyl sulphonyl fluoride (all from Sigma-Aldrich), and 2 μmol/l purified β2-microglobulin (β2m) on ice. Ten-fold dilutions of each peptide were made with 100% dimethyl sulphoxide in 96-well round-bottomed polypropylene plates, then 1 μl of individual aliquots were transferred into new plates and 99 μl of the above HLA-β2m mixture was added to each well (i.e. 100-fold dilution for each peptide solution). The plates were incubated on a shaker at 4°C for 48–72 h. One day before ELISA analysis, 96-well ELISA plates (Costar, Cambridge, MA, USA) were coated with 50 μl/well W6/32 monoclonal antibody (mAb) (10 μg/ml) in 50 mmol/l of carbonate–bicarbonate buffer, pH 9·6 (Sigma, St. Louis, MO, USA) and kept overnight at 4°C. After washing three times with washing buffer containing 0·05% Tween-20 (Sigma) in phosphate-buffered saline (PBS), the wells were blocked for 1 h. Just prior to the ELISA analysis, the reaction volume was diluted 10 times by PBS at 4°C, and 50 μl/well of aliquots were transferred in duplicate to the W6/32 mAb-coated plates. The plates were incubated for 2 h at room temperature, and then washed six times. To detect properly refolded complexes, incubation was for 2 h at room temperature with 100 μl/well of horseradish peroxidase (HRP)-conjugated anti-human β2m mAb (1:1000 dilution; Dako, Copenhagen, Denmark), followed by washing as above. Finally, colour development was performed by ELISA as above.

Reverse transcription PCR analysis of components for antigen processing

A reverse transcription PCR assay was used to examine the expression of LMP2, LMP7, PA28α, TAP1, TAP2 and β-actin (as an internal control) mRNA. Total RNA was extracted using an RNeasy Mini Kit (Qiagen), and cDNA was synthesised by standard methods. Specific primers used were prepared as reported previously (Ito *et al*, 2006). The PCR products were separated in 2·5% agarose and visualised with ethidium bromide staining.

Confocal microscopy

Cells were fixed and permeabilised with Cytofix/Cytoperm (BD Biosciences, San Diego, CA, USA), incubated for 60 min with goat anti-cathepsin H monoclonal and mouse anti-HLA-DM antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then cells were reacted with appropriate fluorescence-labelled secondary antibodies for 60 min. Finally, stained cells were washed and cytocentrifuged, and analysed by laser scanning confocal microscopy using Radiance 2100 K-3 (Bio-Rad, Hercules, CA, USA).

Tetramer construction and flow cytometric analysis

HLA-A*3101 or -A*3303 tetramers incorporating the identified peptides were produced as described previously (Altman *et al*, 1996). 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 fluorescein isothiocyanate-conjugated anti-CD3 (Becton-Dickinson, San Diego, CA,

USA) and Tricolor anti-CD8 mAb (Caltag, Burlingame, CA, USA) on ice for 15 min. Cells were analysed with a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson).

Results

Characterisation of CTL clones

Cytotoxicity assays using a panel of B-LCLs showed that the $CD8^+$ CTL clone 2A10, derived from UPN 027, was HLA-A*3303-restricted, while 1A8, from UPN 028, was HLA-A*3101 restricted (data not shown, and Table 1). Each lysed its respective recipient-derived B-LCLs and phytohaemagglutinin (PHA)-stimulated T-cell blasts, but not donor-derived B-LCLs or natural killer-sensitive K562 cells (Fig 1A and B). Specific lysis of respective recipient B-LCL was inhibited by anti-HLA class I mAb, but not by mAb against anti-HLA-DR, indicating recognition of HLA class I-restricted mHags (Fig 2C and D). No cytotoxicity was observed against the recipient's dermal- and bone marrow-derived fibroblasts even after treatment with IFN-γ and tumour necrosis factor-α (Fig 1E and F). UPN 028 was also HLA-A*3303 positive and his B-LCL were found to be lysed by 2A10 as well. Primary leukaemic blasts from UPN 028 were tested for recognition by the CTL clones. Both 2A10 and 1A8 lysed the leukaemic blasts (Fig 1E and F), indicating that the mHag was expressed on myeloid leukaemic cells. The U937 cell line (Sundstrom & Nilsson, 1976) (subsequently confirmed to be mHag-positive by genotyping) was lysed by both CTL clones only when the respective HLA allele was transduced, confirming HLA-restricted recognition by these CTL clones (Fig 1E and F). Finally, mHag-specific recognition of the U937 cell line was confirmed by cold target inhibition assays. Killing of HLA-transduced U937 was inhibited by the addition of respective recipient B-LCLs or donor LCLs pulsed with the cognate mHag identified in the subsequent study (see below) (Fig 1G and H), while addition of unpulsed donor B-LCLs showed no inhibition. These data suggest that the mHags recognised by 2A10 and 1A8 clones were presented by the U937 cell line. The preferential lysis of haematopoietic cells by these CTLs prompted us to identify the gene(s) encoding the mHags as potential therapeutic targets.

Identification of the gene encoding the mHags as cathepsin H

Two-point linkage analysis was performed to identify the gene encoding the mHags recognised by 2A10 and 1A8, as reported previously (Akatsuka *et al*, 2003b). Surprisingly, the two CTL clones showed identical lytic patterns against CEPH B-LCLs in five families tested (Fig 2A and B, and data not shown), suggesting that the polymorphic genes controlling the expression of the two mHags were located on a narrow chromosomal region or, alternatively, that a single gene encoded both mHags, as seen with the BCL2A1 mHags (Akatsuka *et al*, 2003b).

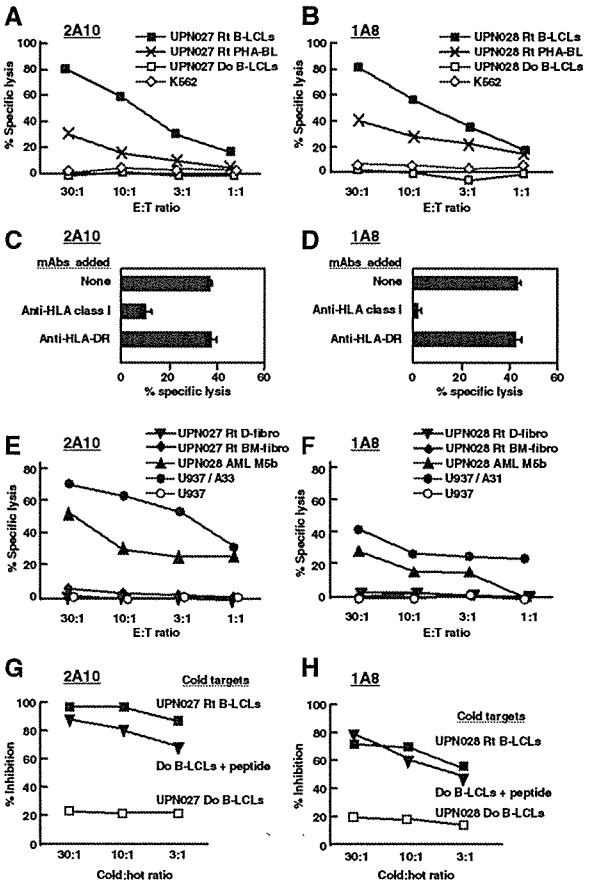

Fig 1. Cytotoxicity of two cytotoxic T lymphocyte (CTL) clones derived from different post-haematopoietic stem cell transplantation recipients. Standard ^{51}Cr-release assays were conducted for the human leucocyte antigen (HLA)-A*3303-restricted CTL clone, 2A10 (A) and the HLA-A*3101-restricted CTL clone, 1A8 (B) against target cells derived from recipient (Rt) B lymphocyte cell lines (B-LCLs), phytohaemagglutinin blasts and donor (Do) B-LCLs at the E:T ratios indicated. Natural killer-sensitive K562 cells were also included as target cells. (C, D) Inhibition of cytotoxicity by anti-HLA monoclonal antibodies (mAbs). Chromium-labelled recipient B-LCLs were incubated with anti-HLA class I mAb (W6/32) or HLA-DR mAb (HDR1), and were tested by CTL clones, 2A10 (C) and 1A8 (D), respectively, at an E:T ratio of 3:1. Cytotoxic activity of 2A10 (E) and 1A8 (F) was also tested against recipient dermal fibroblasts (D-fibro) and bone marrow fibroblasts (BM-fibro), primary acute monocytic leukaemic cells (AML M5b blasts) obtained from patient unique patient number 028 who is positive for both HLA-A*3101 and -A*3303, and U937 cell line, which was found to be minor histocompatibility antigen (mHag)-positive by genotyping, with or without transduction of restriction HLA at the E:T ratios indicated. Both types of fibroblast were pretreated with interferon-γ (500 U/ml) plus tumour necrosis factor-α (10 ng/ml) for 48 h before ^{51}Cr labelling. (G, H) Cold target inhibition of cytolysis of U937/A33 and U937/A31 by 2A10 and 1A8 CTL clones respectively. Cold targets were recipient B-LCLs, donor B-LCL with or without the subsequently identified mHag epitope peptide.

However, because the mapped region contains many genes whose characteristics have not yet been fully elucidated, we could not identify candidate gene(s) by this in silico approach.

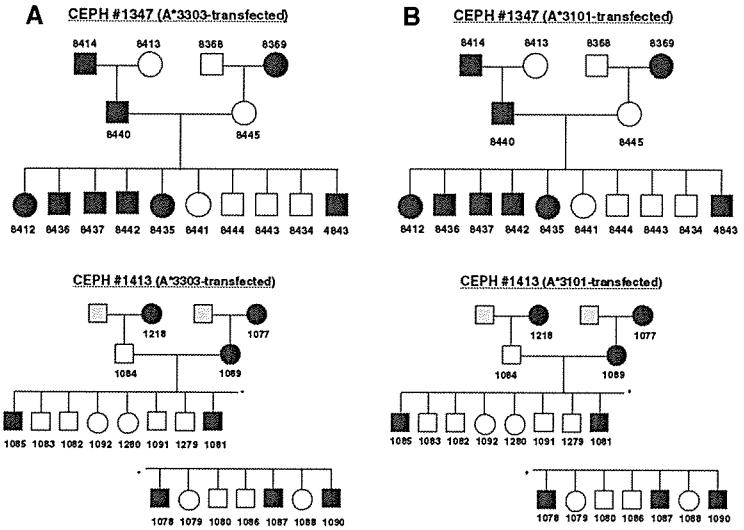

Fig 2. The representative results of phenotyping for the pedigrees of Centre d'Etude du Polymorphism Humain families. B lymphocyte cell lines (B-LCLs) from these families were transduced with human leucocyte antigen (HLA)-A*3303 and -A*3101 and assayed with 2A10 (A) and 1A8 (B) respectively. Filled symbols (■, males; ●, females) represent individuals who were found to be positive in the cytotoxicity assay, and open symbols (□, males; ○, females) represent individuals who were found to be negative. Shaded symbols represent individuals from whom no B-LCL was available. Numbers above or below the symbols were assigned to each family member by the University of Utah (Broman *et al*, 1998).

A cDNA library was generated from UPN 027 B-LCL and used for expression cloning. In the initial screening, one of 96 plasmid pools induced IFN-γ production by 2A10, which was then subcloned into individual cDNA clones and rescreened (Fig 3A). A single cDNA, referred to as B9, was identified that stimulated 2A10 CTL to produce IFN-γ, and recognition of 293T cells required transfection of both HLA-A*3303 and B9 cDNA (Fig 3B, left panel). As the preliminary linkage analysis results showed that the genetic region controlling the mHag recognised by 1A8 was found to be in the same location as that for 2A10 (4·1-Mbp distance around chromosome 15q24–q25, data not shown), we examined whether CTL clone 1A8 might also recognise the B9 cDNA product. Indeed, CTL 1A8 recognised 293T cells expressing B9 cDNA and HLA-A*3101, suggesting that 2A10 and 1A8 CTL clones recognised two different epitopes of the same gene product (Fig 3B, right panel). Subsequently, the cDNA insert of B9 was sequenced and a search of the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) revealed that this cDNA was nearly identical to transcriptional variant 1 encoding isoform a of *CTSH* (GenBank accession no. NM_004390) (Fig 3C), which existed within the region identified by linkage analysis (data not shown).

*Identification of HLA-A*3303 and -A*3101-restricted epitopes on CTSH*

We next sought to identify the polymorphism(s) in CTSH responsible for antigenicity. Splice variant 1 of CTSH mRNA contains three reported non-synonymous coding SNPs (Fig 3C). It was found that the presence of the adenine (translation, Arg) but not guanine (translation, Gly) at nucleotide (nt) position 126 (aa position 11) was associated with recognition by both 2A10 and 1A8 CTL clones. The observed frequency of the adenine-containing allele among 21 Japanese individuals was 0·11.

We then searched for candidate epitope sequences spanning this SNP using on-line algorithms, such as BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind) (Parker *et al*, 1994), and identified nonameric ATLPLLCAR or decameric WATLPLLCAR as candidate epitopes. Both HLA-A*3303 and -A*3101 have Arg or Lys as preferred C-terminal anchor residues, whereas Gly is not a preferred residue (Falk *et al*, 1994; Parker *et al*, 1994; Takiguchi *et al*, 2000). Minigene experiments demonstrated that CTL clone 2A10 recognised 293T/A*3303 expressing WATLPLLCAR but not that expressing nonameric peptide, while 1A8 recognised 293T/A*3101 expressing both ATLPLLCAR and WATLPLLCAR (Fig 3D). Neither CTL clone recognised 293T cells transfected with minigenes encoding the Gly allele.

To determine whether the identified epitopes could sensitise donor HLA-A*3303 or -A*3101-positive target cells to lysis by 2A10 and 1A8, epitope reconstitution assays were performed. As predicted by minigene experiments, 2A10 recognised only the decameric WATLPLLCAR with half-maximal lysis at 2 nmol/l (Fig 4A), although peptide-HLA-binding assays suggested that both nonameric and decameric peptides could bind to HLA-A*3303 equally (Fig 4B). Thus, peptide WATLPLLCAR represents the HLA-A*3303-restricted mHag epitope recognised by 2A10 (designated as $CTSH^R$/A33). 1A8 recognised the nonameric peptide ATLPLLCAR in the context of HLA-A*3101, with half-maximal lysis at 20 nmol/l, and also

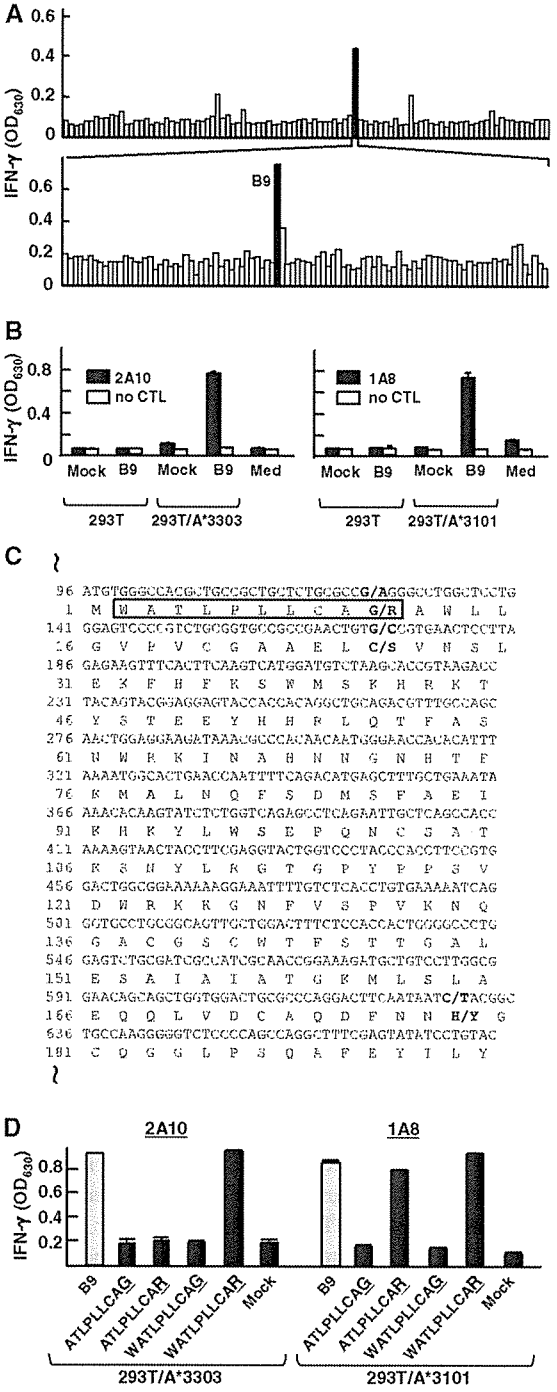

Fig 3. Identification of a cDNA clone encoding the minor histocompatibility antigen (mHag) recognised by cytotoxic T lymphocyte clones 2A10 and 1A8 by expression cloning. (A, upper panel) Screening of cDNA library. cDNA library pools containing 120 cDNA clones were transfected into human leucocyte antigen (HLA)-A*3303 transduced 293T cells (293T/A*3303). After overnight culture with 2A10, interferon (IFN)-γ in the supernatant was measured by enzyme-linked immunosorbent assay. One pool (black bar) was found to stimulate 2A10 efficiently. Subsequently, the pool was divided into individual cDNA clones, then similarly screened. Only 1 cDNA clone, B9, stimulated 2A10 (A, lower panel). The data presented are the OD_{630} mean values for IFN-γ release ± 1 SD of duplicate cultures. (B) Confirmation of the isolated cDNA as the gene encoding the mHags. IFN-γ production in the supernatant of 2A10 stimulated with 293T or 293T/A*3303 transfected with or without cDNA clone B9 (left panel). The error bars indicate the SD from two experiments. IFN-γ production of 1A8 stimulated with 293T or 293T/A*3101 transfected with or without cDNA clone B9 (right panel). (C) Nucleotide and deduced amino acid sequences of the human *cathepsin H* cDNA (NM_004390) and location of mHag epitopes. The deduced amino acids are shown in one-letter designation below the nucleotide sequence. Three previously reported non-synonymous single nucleotide polymorphisms and the corresponding amino acid residues are indicated in bold type. The sequences of identified epitopes are boxed. (D) Identification of the 2A10 and 1A8 epitopes. IFN-γ production of 2A10 (left panel) and 1A8 (right panel) stimulated with restriction HLA-expressing 293T transfected with minigene constructs encoding the nonameric peptide (ATLPLLCAR/G) or the decameric peptide (WATLPLLCAR/G) that were predicted by BIMAS (Parker *et al*, 1994) and SYFPEITHI (Rammensee *et al*, 1999) software. The data presented are the OD_{630} mean values for IFN-γ release ± 1 SD of duplicate cultures. Polymorphic amino acid residues are underlined.

the decameric peptide WATLPLLCAR, but at a 100-fold higher concentration (Fig 4C). However, in this case, the HLA-binding assay demonstrated that almost only the nonameric peptide could bind to HLA-A*3101 (Fig 4D). These data demonstrate that the nonameric peptide ATLPLLCAR represents the HLA-A*3101-restricted 1A8 epitope (designated as $CTSH^R$/A31).

Finally, A31/$CTSH^R$ or A33/$CTSH^R$ tetramers, which were prepared in our laboratory, was able to detect growing populations of $CD8^+$ T cells in T-cell lines generated from UPN 028s post-HSCT PBMC by two different stimulators, recipient pre-HSCT PBMC and mHag peptide-pulsed donor PBMC, indicating that $CTSH^R$ mHags were indeed immunogenic and sensitised precursor T cells were generated in the recipients. However, tetramer-positive cells were not detectable as a distinguishable cluster in the unstimulated $CD8^+$ fraction of any of the post-HSCT samples tested (data not shown), implying that the precursor frequency $CTSH^R$-specific T cells in unstimulated PBMCs was $<10^{-3}$ (data not shown).

Lysosomal localisation of CTSH does not correlate with susceptibility to lysis

Because mHags $CTSH^R$/A31 and $CTSH^R$/A33 are generated only from the isoform a of CTSH that is anticipated to localise to lysosomes, confocal microscopy was used as this approach would make it possible to address the question of whether preferential accumulation of CTSH isoform a in lysosomes correlated with the differential susceptibility to CTL of individual target cells. Haematopoietic cells, including not only monocytes, PHA-blasts, B-LCLs but also most primary leukaemia cells were positive for CTSH, but co-localisation of CTSH with lysosome-resident HLA-DM was not evident