

Synaptojanin 2 is recognized by HLA class II-restricted hairy cell leukemia-specific T cells

EHA Spaenij-Dekking¹, J Van Delft^{2,4}, E Van der Meijden¹, HS Hiemstra², JHF Falkenburg¹, F Koning², JW Drijfhout² and JC Kluin-Nelemans³

¹Department of Hematology, Leiden University Medical Center, The Netherlands; ²Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; and ³Department of Hematology, University Hospital Groningen, Groningen, The Netherlands

Hairy cell leukemia (HCL) is a chronic mature B-cell leukemia characterized by malignant B cells that have typical hairy protrusions. To characterize possible HCL-associated tumor antigens, we generated an HCL-specific and HLA class II (DPw4)-restricted proliferative CD4⁺ T-cell clone. To identify the target antigen of these T cells, we constructed a synthetic peptide library dedicated to bind HLA DPw4, and identified a mimicry epitope recognized by the T-cell clone. With this epitope, the recognition motif of the T-cell clone was deduced and a peptide of human synaptojanin 2 (Syn 2) was identified that stimulated the HCL-reactive T-cell clone. Both Northern and Western blot analyses showed that Syn 2 expression was increased in HCL samples compared to other B cells. Besides, the Syn 2-expressing cell line AML193, with the introduced restrictive HLA-DPw4 molecules, was recognized by the HCL-specific T-cell clone. These results indicate that Syn 2 is a target of autoreactive HCL-specific T cells. Since Syn 2 is a phosphatidylinositol 4,5-biphosphatase involved in cell growth and rearrangement of actin filaments, the increased Syn 2 expression may correlate with the disease etiology or the characteristic morphologic alterations caused by the disease.

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Introduction

Hairy Cell leukemia (HCL) is a chronic B-cell malignancy characterized by splenomegaly, pancytopenia and neoplastic B cells in blood, bone marrow (BM) and spleen.¹ The neoplastic cells contain characteristic hairy protrusions on their surface. It has been shown that these morphologic alterations can be attributed to changes in the organization of the cytoskeleton,^{2–4} for example, the arrangement of F actin with thin microvilli.⁵

The etiology of HCL remains unknown. By epidemiological and cytogenetic studies no correlation between environment and occurrence of the disease was determined and no chromosomal abnormalities characteristic of the disease could be detected.^{6–9} A viral origin has been proposed for several reasons, including the occurrence of tubuloreticular inclusions in hairy cells,^{10–12} the invariable disease pattern and the sensitivity to IFN- α , but no specific agent has been found.

HCL patients do not suffer from the leukemic B-cell proliferation itself but from the opportunistic infections caused by a combination of monocytopenia, pancytopenia, in general, and a severe T-cell dysfunction. Both the number of T cells, the

CD4/CD8 T cell distribution¹³ and T-cell repertoire³ are disturbed. Whereas in healthy individuals T cells are distributed equally over the T-cell receptor V beta chain (TCRBV) families, in HCL patients some TCRBV families are clonally expanded, while others are weakly expressed or even absent.³

Recently, we isolated several HLA class II-restricted CD4⁺ T cell clones that specifically recognized HCL.¹⁴ These T cells have a capacity to proliferate in response to HCL; however, this does not result in an effector mechanism to kill tumor cells efficiently. Therefore, these T cells do unlikely play a role in suppressing the malignancy. In this study, we applied an HLA DPw4 dedicated synthetic peptide library to identify the antigens recognized by an HCL-specific HLA DPw4-restricted T-cell clone. An epitope of synaptojanin 2 (Syn 2), a phosphatidylinositol 4,5-biphosphatase (PtdIns(4,5)P2) involved in cell growth and cell morphology, was found to be recognized by the HCL-specific T cells.

Materials and methods

T-cell clone

The HLA-DPw4-restricted HCL-specific CD4⁺ proliferative T cell was generated by CD40 ligand-activated HCL stimulation of autologous T cells derived from the spleen of a patient with HCL.¹⁴ To ensure clonality of the T cells, recloning was performed by single-well/ single-cell FACS sorting. T cells were cultured by aspecific stimulation with a mixture of irradiated peripheral blood mononuclear cells (PBMCs) as feeder cells, 100 U/ml interleukin-2 (Chiron, Amsterdam, The Netherlands) and 1 μ g/ml phytohemagglutinin (PHA, Murex Diagnostics, Dartford, UK) in Iscove's modified Dulbecco's medium (IMDM, Bio Whittaker, Verviers, Belgium) with 10% pooled normal human AB serum. Identity of the clone was confirmed on genomic DNA by a clone-specific polymerase chain reaction (PCR), using a primer specific for the V β 8.3 region (5'-CCATGATGCGGGGACTGGAGTTGC-3') and a primer annealing in the clone-specific CDR3 region (5'-CTTCCCCCTGTCCAGGACC-3').¹⁴ In this way, 16 clones were obtained originating from the BV8.3 T cell present in the HCL reactive T-cell line. Clonality of the expanded T cells was ascertained by a PCR-based method detecting most of the V α and V β TCR families.

HCL specificity of the clones was ascertained by a proliferation assay using autologous Epstein-Barr virus-transformed B cells (EBV-LCL), and HLA-typed samples of patients with HCL (HCL, $n=3$), B-lineage acute lymphoid leukemia (B-ALL, $n=2$), chronic myeloid leukemia (CML, $n=1$) and peripheral blood lymphocytes (PBLs) of a healthy blood donor as stimulator cells.

The cytokine expression profile of clone V β 8S3⁺ was determined by induction in coating wells of a nontissue

Correspondence: Dr EHA Spaenij-Dekking, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands; Fax: +31 715216751

⁴Passed away before submission

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culture-treated plate with anti-CD3 antibodies (RIV-9, RIVM, Bilthoven, The Netherlands) or with 5 $\mu\text{g/ml}$ Campath (kind gift of Dr G Hale, University of Oxford, UK) as a control (only T-cell binding, no stimulation). T cells (5×10^4) in IMDM supplemented with 10% pooled normal human serum were added and cells were incubated at 37°C. After 24 h, supernatant was collected and the cytokine content was measured using standard Elisa's (CLB, Amsterdam, The Netherlands) according to the manufacturer's procedures.

Cells

The human myelomonocytic leukemia cell line U937 (ATCC CRL-1593.2), the acute myeloid leukemia (AML) cell line AML193 (ATCC CRL9589), the chronic myelogenous leukemic cell line K562 (ATCC-243), the acute T-lymphoblastic cell line MOLT-4 (ATCC CRL-1582) and the cervical adenocarcinoma cell line HeLa (ATCC CCL-2) were obtained from the American Type Culture Collection. The EBV-LCLs of HCL patients were established by *in vitro* transformation of 10^7 spleen cells.¹⁴ Both the EBV-LCL and the ATCC obtained cell lines were maintained in IMDM medium supplemented with 3 mM glutamine and 10% fetal calf serum.

After informed consent, cryopreserved spleen, PBL or BM samples from patients with HCL (spleen, $n=12$), B-ALL ($n=4$), non-Hodgkin's lymphoma (B-NHL, $n=2$), AML ($n=1$) and CML ($n=1$) were used. At least 16 h before an experiment, cells were thawed and incubated at 37°C in IMDM medium supplemented with 3 mM glutamine and 10% pooled human serum.

Design, synthesis and use of a dedicated synthetic peptide library

Hybrid TentaGelH-AM resin (particle size, 90 μm ; loading, 100 pmol/bead, 16 pmol acid stabile attached, 84 pmol acid labile attached)¹⁵ was used to synthesize a random one-bead—one-peptide 14-mer peptide library containing a DPw4 binding motif.¹⁶ The hybrid resin allows for a convergent library screening using the acid cleavable part of the peptide material attached to the resin, combined with efficient peptide identification by Edman sequencing or MS-MS mass spectrometry, using the noncleavable part of the peptide material attached to the resin. The synthesis design of the library is summarized by:

XX(F, L, Y, M)XXXXX(F, L, Y)XX(V, Y, L)XX – Gaba

X being one of the 19 L-amino acids (all natural amino acids except C, which was omitted for synthetic reasons), and Gaba being γ -aminobutyric acid. The library was synthesized using the chemistry described for peptide synthesis following a mix and split protocol,^{17,18,18} yielding a one-bead—one-peptide library with a complexity of 8×10^6 .

Convergent peptide cleavage and screening was performed as described.¹⁵ Briefly, the library was divided into 384 pools of 20 000 beads. Part (about 25%) of the peptide material was released from the beads for testing in a T-cell proliferation assay (first round of screening). Beads of proliferation-inducing pools were subdivided into 288 pools of 70 beads. Again about 25% of the total peptide material was cleaved from the beads for the second round of screening. For the third round of screening, beads were divided into wells containing either 0 or 1 bead/well. The remaining acid labile-attached peptide was released

and tested. Single beads (still containing sequenceable amounts of peptide; 10–15 pmol) were manually put into a cartridge and subsequently sequenced using a Hewlett-Packard G1005A protein sequencer (Palo Alto, CA). As a control, the sequence of the corresponding soluble peptide was determined by MS-MS spectrometry.

Library pools were tested in a quantity of 7 μl /well, giving a final test concentration of 5 nM for each individual peptide in 0.1% DMSO (v/v).

Peptide synthesis

Synthetic peptides were synthesized on a Abimed 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany) using fluorenylmethoxycarbonyl-protected amino acids and TentagelS-AC resins (Rapp, Tübingen, Germany) as described.¹⁹ The purity of the peptides was determined by reversed-phase HPLC, and the integrity of the peptides was determined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy on a Lasermat mass spectrometer (Finnigan-MAT, Hemel Hempstead, UK). Individual peptides were tested at a final concentration of 0.1–1 μM in 0.01–0.1% DMSO (v/v).

Proliferation assays

For characterization of the HCL-specific clone V β 8S3⁺, stimulator cells were either thawed and incubated in IMDM with 10% normal human serum or taken directly from growing cultures. Stimulator cells were irradiated and 10^5 HCL cells, 2.5×10^4 EBV-LCL, or 5×10^4 PBL with or without preincubation with anti-HLA class II-specific antibodies, were cocultured for 3 days with 10^4 V β 8S3⁺ cells in a total volume of 150 μl IMDM containing 10% pooled normal human serum. After 3 days ³H-thymidine (1 μCi /well) was added to the cultures, and 18–20 h thereafter cells were harvested. ³H-thymidine incorporation in the T-cell DNA was counted on a liquid scintillation counter (1205 Betaplate, Liquid Scintillation Counter, LKB Instruments, Gaithersburg, MD, USA).

For the peptide (library and single peptide) screening, 5×10^4 irradiated HLA-DPw4-matched PBMCs (HLA-DPB1*0401 or HLA DPB1*0402) were preincubated with peptide in flat bottomed 96-well plates in 100 μl IMDM containing 10% normal human serum for at least 2 h at 37°C. Next, 10^4 T cells in 50 μl of the same medium were added. After 3 days ³H-thymidine (1 μCi) was added, and proliferation was calculated as described before.

Northern blot analysis

Northern blot analysis was performed according to standard laboratory procedures.²⁰ Syn 2-specific RNA was visualized using a ³²P-labelled 400 bp PCR product of Syn 2 (forward primer, 5'-GCTATGACTGAGCGTCAGTCCG-3'; and reverse primer, 5'-CTTCGTTAGGG AGATCGCGATTTCG-3'). As control for the amount of RNA loaded in each lane, the blot was additionally probed with a ³²P-labelled PCR product for the housekeeping gene β -actin.

Western blot analysis

Western blot analysis was performed according to standard laboratory procedures. Syn 2 was visualized by the Syn 2-

specific polyclonal Ab,²¹ kindly provided by Dr M Symon (Onyx Pharmaceuticals, Richmond, CA, USA).

Construction of retroviral vectors and generation of retroviral supernatant

Both Syn 2 and HLA DPw4 constructs for retroviral expression were made in the previously described bicistronic vectors in which the multiple cloning site is linked to the downstream internal ribosome entry sequence and the marker gene green fluorescent protein (GFP) or the truncated form of the nerve growth factor (Δ NGF-R).²² By reverse transcriptase-PCR on AML-193 cells (a high mRNA expression of Syn 2) different PCR products of Syn 2 were obtained that were cloned in combination with Δ NGF-R. Both HLA DPB1*0401 and HLA DPA1*0103 were amplified from cDNA of HCL cells of the autologous patient of clone $V\beta 8.3^+$ and cloned into retroviral vectors. The HLA-DPA chain was cloned in combination with GFP and the HLA-DPB chain was cloned in combination with Δ NGF-R. Retroviral vectors encoding GFP or Δ NGF-R alone were used as control vectors. The constructs were transfected into ϕ -NX-A cells (kindly provided by G Nolan, Stanford University, Palo Alto, CA, USA) using calcium phosphate (Life Technologies, Gaithersburg, MD, USA), and 2 days later 2 μ g/ml puromycin (Clontech, Palo Alto, CA, USA) was added. At 10–14 days after transfection, 6×10^6 cells were plated per 10-cm Petridish (Beckton Dickinson, Meylan, France) in 10 ml IMDM supplemented with 10% FBS without puromycin. The next day, the medium was refreshed and on the following day retroviral supernatant was harvested, and frozen at -70°C .

Retroviral transduction

For transduction non tissue culture-treated plates (Beckton Dickinson, Meylan, France) were coated with retronectin (Takara, Shiga, Japan) for 2 h at room temperature. Next, the coated wells were blocked with 2% human serum albumin (CLB, Amsterdam, the Netherlands). Then, cells (EBV-LCLs for Syn 2 constructs or AML 193 for HLA constructs) were added, and after 30 min, culture medium was removed, and thawed retroviral supernatant was added. Cells together with viral supernatant were incubated overnight, washed and finally

transferred to cell culture bottles in normal tissue culture medium. After 3 days, transduced cells were sorted by fluorescence-activated cell sorting on the basis of reporter protein expression. After 2 weeks of culture, from the GFP⁺ and/or Δ NGF-R⁺ positive population AML-193 cells with high expression of HLA DPw4 were sorted on an FACS Vantage (Becton Dickinson, Mountain View, CA, USA), and used in the experiments. For EBV-LCL cells containing the Syn 2 constructs, the Δ NGF-R⁺ positive populations were used in the experiments.

Results

Cloning, expansion and characterization of $V\beta 8.3$ -T-cell clone from HCL reactive T-cell line C6

The previously described T-cell clone $V\beta 8.3^{14}$ was re-cloned under conditions that ascertain expansion of a clonal T-cell population. HLA restriction and HCL specificity were reconfirmed as described before (Figure 1).¹⁴ Clonality of the expanded population was confirmed. The cytokine profile established by anti-CD3 stimulation showed secretion of IL-4 (>900 pg/ml), GM-CSF (>150 pg/ml), TNF- α (>250 pg/ml) and IFN- γ (>400 pg/ml).

Synthesis and screening of an HLA DPw4 binding, one-bead-one-peptide synthetic peptide library

A 14-mer one-bead-one-peptide synthetic peptide library^{19,23,23} consisting of about 8×10^6 peptides was synthesized. The peptides in this library were enriched for potential HLA-DPw4 binding peptides by including amino acids at anchor positions that enhance binding of a peptide to HLA DPw4.¹⁶ The library was screened using the three-step converging screening system as described.¹⁵ In the initial screening round, two peptide pools induced proliferation (SI of 10 and 20, respectively). The peptide-bearing beads from the pool with the highest induction of proliferation were used in the second round of screening. In this round again one peptide pool induced proliferation (SI >20). In the last round consisting of single peptides, only one induced proliferation (SI = 10), and the corresponding was subjected to Edman sequencing and mass spectrometric analysis

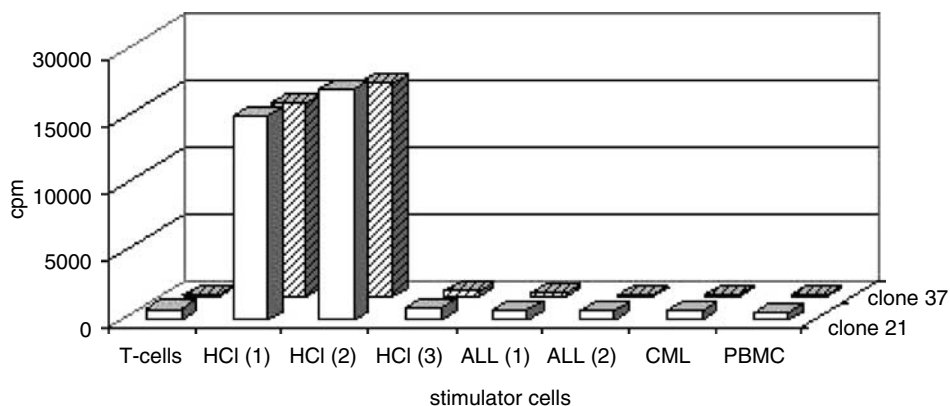


Figure 1 Representative example of HCL-specific recognition by BV8.3-positive T-cell clones isolated in this study. Proliferation of the BV8.3⁺ T-cell clones 21 and 37 was measured in response to malignant HLA DPw4-matched cell samples: HCL (1 and 2), ALL (1 and 2) and CML, and to a malignant HLA DPw4 mismatched cell samples HCL (3). As a control proliferation was measured in response to PBMCs of a healthy HLA DPw4-matched donor.

to determine its amino-acid sequence. The results of both techniques yielded the sequence of the mimicry epitope EFYYEPFAYVVVVV.

Definition of the recognition motif and database analysis

We have previously shown that the sequence of a mimicry epitope can be used to determine a recognition motif for a T-cell clone. This recognition motif can be used to search protein databases to identify the putative natural ligand of the T-cell clone. To define the recognition motif of clone V β 8S3⁺, 14-mer peptides were synthesized, comparable with the mimicry epitope, in which one of the 10 central amino acids was substituted for all of the remaining 19 amino acids (cysteine was excluded). The resulting 190 peptides were screened for recognition by clone V β 8S3⁺. Sequences of peptides that were recognized at a 50–100% level compared to recognition of the mimicry epitope were used for the definition of the recognition motif; X-X-[YFILMRW]-[YFIW]-[EFW]-[PTVY] X-X-Y-X-V-[VPW]-X-X. With this motif, Swissprot and TrEMBL protein databases were searched resulting in a total of 38 hits (seven hits out of 85 785 entries in the Swissprot database and 31 hits out of 2 99348 entries in the TrEMBL database). Out of these 38 possible epitopes, only 14 were encoded by human proteins or by proteins of organisms known to infect humans (Table 1). These 14 epitopes were synthesized and tested for recognition by clone V β 8S3⁺. Only the epitope CLYIFVRPYHVPFI, encoded by human Syn 2, was recognized.

Expression of human Syn 2 in various cell lines and hematologic malignancies

To investigate the expression of Syn 2 at mRNA level, RT-PCR was performed on samples from both HCL cells and EBV-LCL from three HCL patients. The presence of the expected 800 base pair (bp) from the Syn 2-specific PCR fragment could be demonstrated in HCL cells from all three patients. In one of the EBV-LCL samples Syn 2 expression could also be demonstrated (not shown).

In an attempt to quantify expression of Syn 2 in HCL, EBV-LCL and other cell types, Northern blot analysis was performed with samples of various leukemic cell lines and hematologic malignancies (Figure 2). Syn 2 expression was clearly found in

HCL cells and in the AML cell line AML-193. Weaker expression was found in the T-ALL cell line Molt-4, and in fresh AML cells. In U937, K562, EBV-LCL, B-lineage ALL and B-NHL hardly any expression of Syn 2 was found. By Western blot, Syn 2 protein expression was found in all 12 different HCL samples tested (Figure 3a). In addition, similarly to the expression seen at mRNA level, Syn 2 was also present in Molt-4 and in the AML193 cell line, although only smaller protein fragments could be detected by the Syn 2-specific antibodies. Surprisingly, in this experiment also one of the tested EBV EBV-LCL showed Syn 2 expression. When tested in a proliferation assay this EBV-LCL was recognized by clone V β 8S3⁺ (result not shown). In HeLa, K562, B-lineage ALL, B-NHL and AML cases no expression of Syn 2 was found (Figure 3b).

Viral transduction of Syn 2 and HLA DPw4

To investigate whether Syn 2 is the target of clone V β 8S3⁺, different retroviral vectors were constructed. These constructs included Syn 2 together with the N-terminal signal peptide, Syn 2 without signal peptide, a 552 N-terminal deletion mutant of Syn 2 and protein fragments of different lengths all including the epitope recognized by clone V β 8S3⁺. All constructs were made in bicistronic vectors linking RNA synthesis of the Syn 2 constructs via a downstream ribosome entry sequence to the

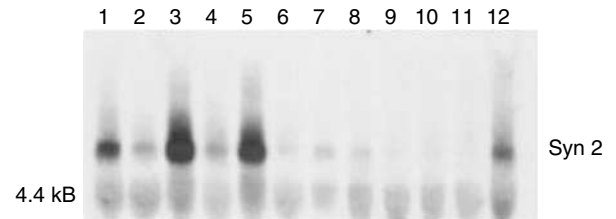


Figure 2 Northern blot analysis of Syn 2 expression in various cell lines and hematologic malignancies. For each sample, RNA was isolated from 10×10^6 cells, of which $20 \mu\text{g}$ was separated on a 2% agarose gel. RNA was blotted on nitrocellulose followed by hybridization of Syn 2 RNA with a 800 bp ^{32}P -labelled specific probe and visualized by autoradiography. Lane 1, Molt-4; lane 2, K562; lane 3, AML-193; lane 4, U937; lane 5, HCL pat A; lane 6, EBV-LCL pat A; lane 7, EBV-LCL pat B; lane 8, B-ALL pat A; lane 9, B-ALL pat B; lane 10, B-NHL pat A; lane 11, B-NHL pat B; lane 12, AML pat A.

Table 1 Peptides of different origin (human, viral and bacterial) that might encode the HCL-specific epitope of clone V β 8.3

Peptide sequence	Protein description and origin
1. QPWYFTTTYNVVVKP	Cellular retinaldehyde binding protein (human)
2. QELIEYDTYKVVVC	Putative polyketide synthetase pKSL (<i>Bacillus subtilis</i>)
3. TWFIFLTYTVVVPN	Ribonuclease BN (<i>Haemophilus influenza</i>)
4. IKYIFVAGYLWYD	Capsid protein infectious laryngotracheitis virus (ILTV)
5. TRIYETDKYVWVDV	Hypothetical protein (<i>Methanococcus jannischii</i>)
6. CLYIFVRPYHVPFI	Synaptojanin 2 (human)
7. QPWYFTTTYNVVVKP	Retinaldehyde binding protein (<i>Ateles belzebuth chamek</i>)
8. TNMYFVYDYNWVDC	ORF F264 (<i>Escherichia coli</i>)
9. KRLLFPFFLYLVVN	TASA protein (<i>Streptococcus pneumonia</i>)
10. NSFIEYPQYTPVWLL	Putative lipoprotein (<i>Mycoplasma pneumonia</i>)
11. KKLIEVATYVWLL	ORF 348 protein (<i>Lactococcus lactis</i>)
12. NDYYEVFNYYVVIT	Hypothetical protein (<i>Streptococcus aureus</i>)
13. VKYIEYGVYIVVGI	GP41 (Human immunodeficiency virus-2)
14. AERIFYATYSVWTD	Serine/threonine protein kinase (<i>Chlorella virus</i>)

The epitopes were the result of Swissprot and TrEMBL protein database searches using the recognition motif of clone V β 8S3⁺ as a lead.

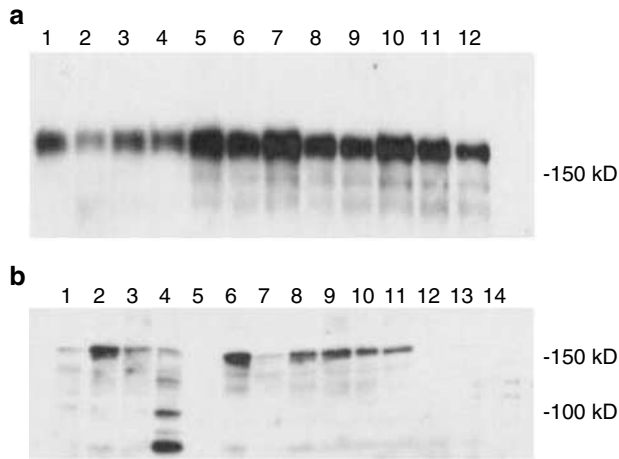


Figure 3 Syn 2 expression was determined by Western blot analysis. For each sample protein extracts were made of 5×10^6 cells, of which $25 \mu\text{g}$ of total protein was separated on a 6.5% denaturing SDS-PAGE gel. Next, proteins were blotted on nitrocellulose membranes and the presence of Syn 2 was shown using specific polyclonal antibodies.²¹ (a) Syn 2 expression in HCL material of 12 different HCL patients. (b) Syn 2 expression in various cell lines and hematologic malignancies. Lane 1, HeLa; lane 2, Molt-4; lane 3, K562; lane 4, AML 193; lane 5, U 937; lane 6, HCL pat A; lane 7, EBV-LCL pat A; lane 8, HCL pat B; lane 9, EBV-LCL pat B; lane 10, B-ALL pat A; lane 11, B-ALL pat B; lane 12, B-NHL pat A; lane 13, B-NHL pat B; lane 14 AML pat A. For cell line AML193 also smaller protein fragments could be detected, probably protein products of alternatively spliced Syn 2.

marker gene $\Delta\text{NGF-R}$.²⁴ Autologous EBV-LCLs were transduced with either the different Syn 2 constructs or the control construct only expressing $\Delta\text{NGF-R}$. After transduction, EBV-LCLs positive for $\Delta\text{NGF-R}$ were sorted by fluorescence-activated cell sorting. Although mRNA was transcribed in these cells, illustrated by the reporter gene. By Western blot analysis no significant amounts of Syn 2 or protein fragments could be detected in any of the transduced EBV-LCL cell lines (result not shown). When tested for recognition by clone $V\beta 8\text{S}3^+$, the T-cell clone recognized none of the transduced EBV-LCLs.

In addition to HCL cells, Syn 2 appeared highly expressed by the leukemic cell line AML-193. This cell line, which had a $\text{DPB}0^*101$, $\text{DPB}1^*704$ HLA type, was not recognized by clone $V\beta 8\text{S}3^+$. Therefore, retroviral vectors were constructed in which expression of HLA $\text{DPA}1^*0103$ was linked to the expression of GFP, and HLA $\text{DPB}1^*0401$ was linked to $\Delta\text{NGF-R}$. AML-193 was transduced with both HLA $\text{DPA}1^*0103$ and HLA $\text{DPB}1^*0401$. AML-193 cells positive for GFP and $\Delta\text{NGF-R}$ were sorted and tested for recognition by clone $V\beta 8\text{S}3^+$. As a negative control, the AML-193 transduced with the vectors only expressing the reporter genes were used. The results demonstrated that the AML-193 cells transduced with both the HLA DPw4 genes were recognized by clone $V\beta 8\text{S}3^+$, whereas the parental AML-193 cell line expressing HLA $\text{DPB}0^*101$, $\text{DPB}1^*704$ was not (Figure 4).

Discussion

For the identification of HLA class II-specific antigens recognized by $\text{CD}4^+$ T cell clones, only a few methods have proven to be successful, for example, the cDNA library,^{25,26} the peptide elution²⁷⁻²⁹ and peptide library methods. Since both the cDNA library and peptide elution methods make use of materials of

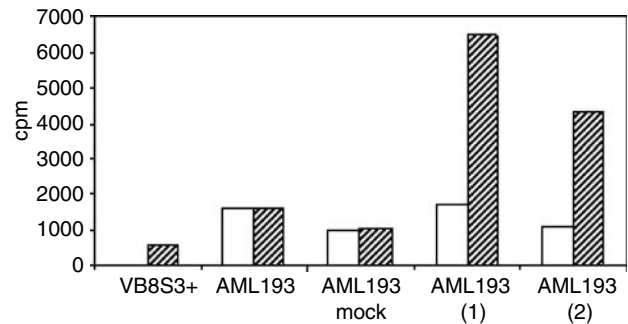


Figure 4 Recognition of AML193 by clone $V\beta 8\text{S}3^+$ after transduction with HLA DPw4 Clone $V\beta 8\text{S}3^+$ was incubated for 3 days with wild-type AML-193, AML-193 mock transfected, two AML-193 lines transduced with HLA $\text{DPA}1^*0103$ and HLA $\text{DPB}1^*0401$ (both transduced lines were made independently). The proliferation of the T cells alone were shown as a control. The open bars represent background proliferation (^3H -thymidine incorporation) given by the irradiated target cells. The hatched bars show proliferation of clone $V\beta 8\text{S}3^+$ after preincubation with the target cells for 3 days.

cells expressing the epitope recognized by the T-cell clone, the epitopes identified will be naturally occurring epitopes. In this study, a synthetic peptide library^{30,31} was used for the identification of the HCL-specific antigen. A possible disadvantage of this synthetic approach might be that the epitopes identified by the library method are no natural epitopes. In previous studies, however, the synthetic epitopes formed valuable leads for identification of natural epitopes.^{19,23,32}

The 14-mer peptide library was synthesized by utilizing the known binding motifs of peptides presented by HLA DPw4,¹⁶ to increase the effectiveness of the library. Out of 14 candidate epitopes of human, bacterial or viral origin, only the epitope encoded by human Syn 2 was recognized. The expression of Syn 2 in HCL cells could be shown at both transcriptional and protein expression level by PCR, Northern and Western blot analyses. However, the expression of Syn 2 is not HCL specific, since at mRNA and/or protein level relatively high expression could also be shown in different cell lines like, Molt-4, AML193, an EBV-LCL and in materials of a patient suffering from AML. Different constructs for viral transduction have been made in an attempt to increase the expression of Syn 2 or of the epitope of Syn 2 recognized by clone $V\beta 8\text{S}3^+$ in the autologous EBV-LCL. However, although the reporter genes were expressed, no expression of recombinant Syn 2 or epitopes of Syn 2 could be demonstrated. Therefore, we have no direct evidence of Syn 2 being the natural epitope of clone $V\beta 8\text{S}3^+$. However, indirect evidence directed to Syn 2 as the natural epitope of the HCL-specific T-cell clone $V\beta 8\text{S}3^+$: first, an EBV-LCL with increased Syn 2 expression was recognized by the T-cell clone, and secondly the transduced cell line AML-193 (constitutive high Syn 2 expression), with the transduced restrictive HLA DPw4 molecules was specifically recognized by clone $V\beta 8\text{S}3^+$.

Syn 2 is a member of the type II phosphoinositol-5 phosphatase family, which also includes Syn 1^{33,34} and three yeast 5-phosphatases (for review, see, Takenawa *et al*³⁵). The proteins consist of three major domains, an N-terminal Sac I domain, a central domain with 5-phosphatase activity³⁶ and a C-terminal proline-rich domain. The Sac I domain functions as a regulatory domain for secretion and actin cytoskeletal reorganization.^{34,37} The central domain is responsible for the 5'-phosphatase activity, whereas the C-terminal proline-rich tail can bind to different SH3 domain-containing proteins. By

binding to such proteins both Syn 1 and Syn 2 probably are involved in various normal and pathogenic pathways. By binding to Grb-2, a member of a family of adaptor proteins that couple receptor tyrosine kinases to the Ras signaling pathway, Syn 2 plays an important role in regulating actin filaments by controlling PtdIns 4,5-P2 levels bound to cytoskeleton-regulating molecules.³⁴ Moreover, Syn 2 assumably regulates cell proliferation by binding to the small GTPase Rac, which inhibits endocytosis of growth factor receptors.^{21,38} An important role of Syn 1 in mixed lineage leukemia (MLL) gene-mediated leukemogenesis is suggested since Syn 1 interacts with the cytoplasmatic proteins EEN and Abi-1,^{39,40} both involved in endocytosis and both being fusion partners of MLL.³⁹ Syn 2 is expressed both in the brain and in the peripheral tissues. Like Syn 1, Syn 2 is subjected to alternative splicing at the C-terminus resulting in RNA transcripts and proteins of different length.^{33,37,41,42} This alternative splicing has probably resulted in the smaller protein fragments that appeared in the Western blot analysis of the AML193 cell line (Figure 3b, lane 4). Syn 1 is expressed in bone marrow and immature hematopoietic progenitor cell lines, including HL60 (promyelocyte), K562 (early erythrocyte) and Molt-4 (immature T cell), but not in Raji (mature B cell); it was weakly expressed in peripheral blood leukocytes, showing that the expression of Syn 1 is differentially regulated during hematopoiesis.³⁹

We determined the expression of Syn 2 both by Northern and Western blot analysis. In contrast to the expression of Syn 1, Syn 2 did not show a differential regulation since it was expressed both in early progenitor cell lines like AML-193, and Molt-4 as in more mature cells like HCL cells and an EBV-LCL.

We can speculate on the function of Syn 2 in either the morphology or pathology of HCL. A hallmark of HCL is the striking morphology of the cells. In the cells, F-actin is associated with thin microvilli responsible for the 'hairy' appearance.⁵ By hydrolysis of PtdIns(4,5)P2 bound to actin-regulatory proteins Syn 2 influences the role of actin. Therefore, Syn 2 might indirectly play a role in the hairy appearance of HCL cells. Moreover, because Syn 2 binds to SH3 domain-containing proteins like Grb2, actin-regulating proteins like α -actinin and probably also to MLL fusion proteins, this suggests that Syn 2 plays a role in the malignant transformation of different cells.^{35,39} Future studies will involve the determination of the exact localization of Syn 2 in a wide variety of cell suspensions and tissues by Syn 2-specific antibodies and analysis of the signal transduction route in which Syn 2 is involved. Therefore, by exploring the function of Syn 2 in HCL, we will hopefully reveal its role in either the characteristic morphology or pathology of the disease.

In conclusion, using the synthetic peptide library approach, Syn 2 was identified as a candidate for the natural epitope of the HCL-specific T-cell clone V β 8S3⁺. Although the expression of Syn 2 in HCL cells is relatively high compared to the expression in other malignancies, the expression is not unique for HCL cells. Given the role of Syn 2 in actin and cell proliferation regulation, it is a tentative candidate for a role in HCL morphology or pathogenesis.

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