

ACTIVATED HUMAN T LYMPHOCYTES EXPRESS MHC CLASS I HEAVY CHAINS NOT ASSOCIATED WITH β_2 -MICROGLOBULIN

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Class I MHC molecules associated with β_2 -microglobulin (β_2m) are peptide-binding proteins on the surface of nearly all nucleated cells and present antigens to CTL (1). HLA class I molecules may also associate with other surface proteins (2-4) and exert a signal-transducing function in these aggregates.

The genes coding for the MHC molecules are the most polymorphic loci known in higher vertebrates. It is generally held that the noncovalent association between the monomorphic light chain β_2m is essential for the transport of the class I molecule to the surface and that surface expressed HLA molecules are linked to β_2m (1, 5).

Here we report that in vitro or in vivo activated, but not resting T lymphocytes, express a considerable number of surface HLA class I heavy chain molecules not associated with β_2m . The conformational properties of such class I molecules unlinked to β_2m (5, 6) are known to be very different from peptide binding, β_2m -associated heavy chain molecules. It is therefore likely that the loss of the β_2m and the conformational alteration of this class I protein upon T cell activation have some important functional implications.

Materials and Methods

Monoclonal Antibodies. The CD25 (clone 3G10) and LA45 antibodies were raised against HUT102 cells in our laboratory; the CD3 antibody OKT3 was purchased from Ortho Pharmaceuticals, Raritan, NJ. The antibody W6/32 (7) reacts with a monomorphic determinant of human HLA-A,B,C molecules and was purchased from Sera-lab, Sussex, UK, and the anti- β_2m antibody (clone SV393) was from Behring, Marburg, FRG. HC10 was a gift of H. Ploegh (Netherlands Cancer Institute, Amsterdam) (8).

Stimulation and Fluorescence Staining of Lymphocytes. Mononuclear cells (MNC) from healthy donors and patients were isolated by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation and either freshly analyzed or cultured for 3 d with 2 mg/ml PHA (Wellcome, Beckenham, UK) in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% FCS (Flow Laboratories, Beckenham, UK) or 10% human AB serum (gave no difference), L-glutamine, and antibiotics. Cells were double stained with antibodies CD3-PE, LA45-FITC,

This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich.
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and CD25-FITC by standard immunofluorescence techniques. The binding of fluorochrome-labeled antibodies was evaluated on a FACS 440 cell sorter (Becton Dickinson, Sunnyvale, CA).

DNA Cloning and Sequence Analysis. mRNA from HUT102 cells were enriched three times with oligo(dT)-cellulose (Pharmacia). cDNA cloning was performed following the protocol of Gubler and Hoffmann (9). After linker ligation, the cDNA was ligated in the Eco RI site of λ ZAP vector (Stratagene, La Jolla, CA, reference 10). The phage library was screened with the mAb LA45 according to the manual of the Protoblot Kit (Promega-Biotech, Madison, WI). Positive clones were excised to pBLUESCRIPT SK⁻ plasmid with the helper phage R408 (both Stratagene). Positive inserts were ligated in the Eco RI site of the Okayama Berg vector (pcEXV3, reference 11) and transfected into COS7 cells using a method described by Aruffo and Seed (12). Transfected cells were analyzed by standard protocol for immunofluorescence staining. Sequencing was done by dideoxycytosine termination (13) using restriction fragment clones, T3 and T7 primers, and the Sequenase Kit (U. S. Biologicals, Cleveland, OH). Data bank search was done with the FASTN algorithm (14) to compare the DNA sequence with the EMBL data bank (Version 17.0, released November 1988), and with the FASTP algorithm (14) to compare the NBRF (Version 15.0, released May 1988) and SWISS-PROT (Version 11.0, December 1989) data banks.

Immunoprecipitations and Western Blot Experiments. Immunoprecipitations were carried out as described elsewhere (15). Briefly, 2×10^8 cells were labeled with Na¹²⁵I (New England Nuclear, Boston, MA) with 20 U lactoperoxidase (Sigma Chemical Co., St. Louis, MO) in 0.003% H₂O₂ and then lysed with 0.5% NP40. Cell lysates were incubated five cycles for 2 h with the first antibody and overnight with the second antibody (sixth round). The antibodies LA45, W6/32, and HC10 were linked to protein A-Sepharose (Pharmacia). For Western Blots, SDS-PAGE (16) separated proteins were electrophoretically blotted to nitrocellulose (Schleicher & Schuell, Dassel, FRG). Protein bands were either detected with biotinylated LA45 antibody and extravidin alkaline phosphatase (bioMakor, Rehovot, Israel), or with LA45 antibody and the Protoblot kit from Promega-Biotech.

Results

Expression of LA45 on In Vitro and In Vivo Activated T Lymphocytes. Freshly isolated MNC of 18 volunteers weakly expressed LA45 in $5 \pm 4\%$ as analyzed by gating for lymphoid cells in flow cytometry (Fig. 1 A). Isolated monocytes ($n = 3$), granulocytes ($n = 2$), and bone marrow MNC ($n = 1$) were negative (<2%). T lymphocytes from 12 healthy individuals activated with PHA became clearly LA45⁺ within 24 h after stimulation with a maximum expression on days 3–6 in a range of $65 \pm 19\%$ (day 3). Fig. 1, B and D, show 3-d PHA-activated T cells of one experiment with 79% LA45⁺ cells and 93% CD25⁺ cells, respectively. For in vivo activated cells, we tested freshly isolated blood lymphocytes from patients with infectious mononucleosis and found in two of three cases CD3, CD8, and HLA class II-positive cells expressing LA45 antigen. One patient showed 84% LA45⁺ T cells (Fig. 1 C), the other 14% LA45⁺ T cells.

Determination of the Molecular Weight of LA45. The molecular mass of the LA45 antigen as determined by immunoprecipitation and Western Blot techniques turned out to be 90/45 kD under nonreducing and 45 kD under reducing conditions (for immunoprecipitation: Fig. 4, lanes *e* and *i*; for Western Blot: Fig. 5, lane *c*). The slight signal of about 38 kD (Fig. 4, lanes *e* and *i*) is co-precipitated by LA45 antibody in some experiments.

Cloning and Sequence Analysis of LA45. To clone the gene encoding LA45 we created a cDNA library of the target cell line HUT102 in the prokaryotic expression vector λ ZAP. By plaque screening with the mAb LA45 we got seven positive clones. We then subcloned these cDNA clones in the eukaryotic expression vector pcEXV3,

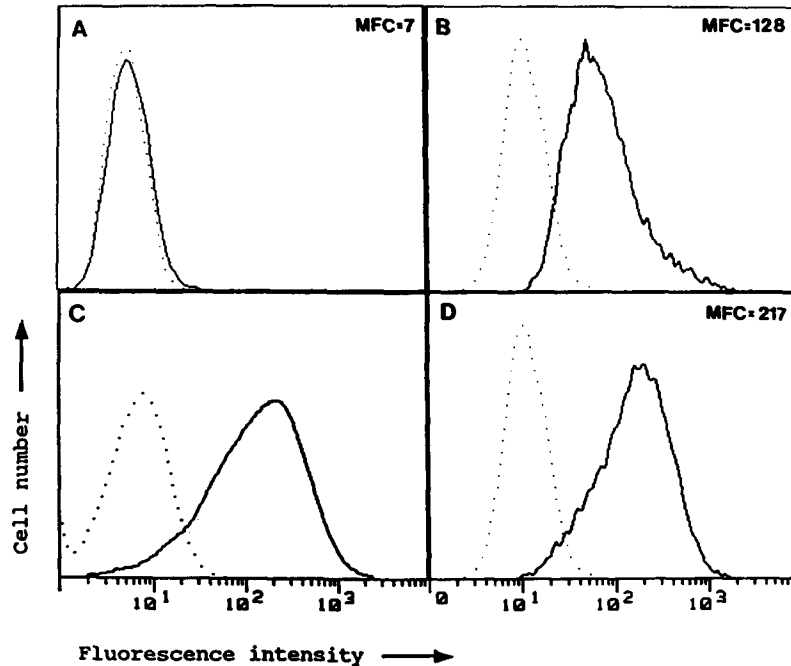


FIGURE 1. Fluorescence staining of human T lymphocytes. (A) Normal resting CD3⁺ cells stained with LA45; (B and D) PHA-activated CD3⁺ cells stained with LA45 (B) and CD25 (D); and in vivo activated, CD3⁺ cells from a patient with infectious mononucleosis stained with LA45 (C).

and expressed the recombinant protein in COS7 monkey cells. The expression of the rLA45 protein was detected in a high amount in the cytoplasm of the COS7 cells, but was only slightly expressed on the cell surface. However, we could demonstrate, both with metabolically [³⁵S]methionine and surface ¹²⁵I-labeled cells, that the transfection-induced recombinant protein has the same size as that expressed by the target cell line HUT102. An immunoprecipitation of recombinant LA45 from ¹²⁵I-labeled transfected COS cells is shown in Fig. 2.

We then sequenced the gene and found a nucleotide sequence of 1418 bases including an open reading frame coding for a protein of 367 amino acids (Fig. 3 A). A putative deletion, indicated as #, is assumed at position 54 to 55 in the nucleotide sequence, leading to a shift in the reading frame in the peptide encoded 5' to this deletion. This part of the protein is coding for a highly hydrophobic leader peptide as is known for membrane transported proteins.

The comparison of the nucleotide and protein sequence of LA45 with data banks revealed that LA45 is quite homologous to genes of the HLA class I gene family. In particular, there is a homology of 92% to the cDNA sequence of HLA-A2 sequence over a range of 1306 nucleotides. At the protein level, LA45 is 95% homologous to the protein sequence of the HLA-Aw68 over a range of 275 amino acids and of 93% homologous to the protein sequence of HLA-A3 over 367 amino acids.

The highly conserved five cysteine residues within the HLA class I genes as well

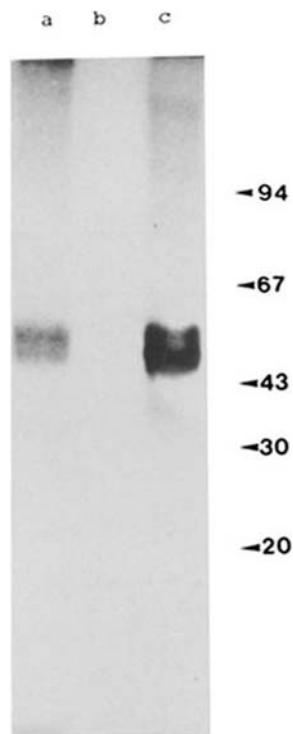


FIGURE 2. Immunoprecipitations of ^{125}I -labeled COS7 cells (12% SDS-PAGE). (lane *a*) LA45-transfected COS7 cells, (lane *b*) untransfected COS7 cells, (lane *c*) HUT102 cells. From all cell types precipitations were done with LA45 antibody. Molecular masses shown in kilodaltons.

as the *N*-glycosylation site (gln-85) of HLA class I proteins were found. The hydrophobicity analysis revealed the typical transmembrane region (aa 303–331). Therefore, we separate the protein sequence according to class I proteins in the domains $\alpha 1$, $\alpha 2$, and $\alpha 3$, transmembrane, and cytoplasmic tail. The direct comparison of LA45 to human HLA class I proteins is seen in Fig. 3 *B*. It is noteworthy that LA45 and HLA-A3 differ only in 23 amino acids.

Comparison of LA45 with HLA Class I Proteins. The high homology of LA45 to HLA class I genes on the one hand and the clearly discrepant cellular expression pattern on the other hand led us to compare HLA class I proteins and the LA45 in a direct manner. Therefore, we performed sequential immunoprecipitation studies with the antibody LA45 and the antibody W6/32, reactive with a monomorphic determinant of HLA-A,B,C molecules. Clearing a lysate of HUT102 cells with LA45 antibody revealed that HLA class I and β_2m proteins remained (Fig. 4, lanes *e–h*). On the other hand, clearing with W6/32 left behind LA45-reactive structures (Fig. 4, lanes *a–d*). The antibody W6/32 precipitated bands at 90 kD and 45 kD under nonreducing conditions and a band of 45 kD under reducing conditions plus β_2m , a protein of 12 kD (lanes *a* and *k*). However, the LA45 antibody reacts with proteins of 90 and 45 kD (nonreduced) and 45 kD (reduced) and does not co-precipitate β_2m (lanes *e* and *i*).

In view of these data one may argue that a possible weak association between LA45 and β_2m was not detected in immunoprecipitation experiments. However, we could

also demonstrate that antibody-induced capping of LA45 did not induce capping of W6/32 or anti- β_2m reactive structures, nor did W6/32 or anti- β_2m -induced capping lead to cap formation of LA45.

Reactivity of LA45 in Western Blots. To check if LA45 antibody reacts with conformationally altered class I heavy chains, we tested a denatured total cell lysate from lymphocytes, which rarely express LA45, and found a clear signal with LA45 antibody in the Western Blot (Fig. 5, lane *b*) at 45 kD. The same signal is found with cell lysates of HUT102 cells, but LA45 antibody was negative in a Western Blot with the cell line K562, which is known to be HLA class I-negative. Furthermore, we isolated conventional HLA-A,B,C molecules from resting, LA45⁻ T cells with W6/32 antibody, denatured them with SDS, and analyzed the PAGE-separated bands with LA45 antibody. As seen from Fig. 5 (lane *a*), such denatured material reacts with LA45 antibody at 45 kD.

Sequential Immunoprecipitation with HC10. The reactivity of LA45 with denatured HLA class I heavy chains was further supported by a sequential immunoprecipitation with the antibody HC10 raised against and reactive with SDS denatured, free class I heavy chain molecule (8). Clearing of lysates from ¹²⁵Iodinated HUT102 cells with HC10 removed all LA45 reactive material (Fig. 6 *A*). As a control for this experiment, we included a sequential immunoprecipitation with HC10 and W6/32. It can be seen from Fig. 6 *B* that HC10 does not precipitate HLA class I proteins associated to β_2m and recognized by W6/32 antibody. Our results thus strongly suggest that activated T cells and possibly other cells express HLA-A,B,C molecules not associated with β_2m .

Discussion

In our attempt to define new activation-induced cellular interaction molecules on human T cells, we raised a mAb (LA45) against the HTLV-1 transformed human T cell line HUT102. The LA45 antigen is found on activated human T lymphocytes with a molecular mass of 90/45 kD (nonreduced) and 45 kD (reduced) (Fig. 4, lanes *e* and *i*; Fig. 5, lane *c*).

Molecular cloning and sequence analysis revealed a high homology of LA45 cDNA and protein sequence to human HLA class I genes. When comparing the LA45 sequence with the grouped HLA class I DNA and protein sequences by Parham et al. (17, 18) we detected all characteristics of A-ness in our cDNA sequence. We found the 62 A-locus characteristic nucleotides as well as the two A-specific methionines (met-138 and met-189) (Fig. 3).

However, HLA class I proteins are known to be expressed on all nucleated cells, but for LA45 antigens we repeatedly showed that they are virtually absent from the surface and cytoplasm of resting peripheral blood and bone marrow mononuclear and polymorphonuclear cells. After stimulation with PHA we can induce the expression of LA45 antigen on T cells within 24 h. The kinetics of the expression of LA45 antigen on T lymphocytes is rather heterogenous when compared with the classical activation antigen CD25. However, as shown by immunofluorescence (Fig. 1, *B* and *D*), the number of these newly expressed structures is essentially comparable to the number of low affinity IL-2 receptors on activated T cells (25,000–60,000 binding sites, reference 19).

A

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1 CATGGCGTCAATGGGCCCCGAACCCCTGCTCTACTCTCGGGGGCCCTGGC*CTGACCCAGACCTGGCGGGCTCCGACTGGATGAGGTATTCTACACC
1 M A V M A P R T L V L L L S G A L # L T Q T W A G S H W M R Y F Y T
104 TCCGTGTCCCGCCCGCGGGGAGCCCGCTTCATCGCGTGGCGTACGTGGAAGACAGCAGTTCGTGCGGTTCCGACAGCGAGCCCGGAGCCAGAGG
35 S V S R P G R G E P R F I A V G Y V D D T Q F V R F D S D A A S Q R
206 ATGGAGCCGCGGGCCCGTGGATAGAGCAGGAGGGCCCGAGTATTGGACCCGAAACACCGAATGTGAAGCCOCAGTCACAGACTGACCGAGTGGACCTG
69 H E F R A P W I E Q E G P E Y W D R N T R N V K A Q S Q T D R V D L
308 GGGACCCCTGGCGGCTACTACAACCAGAGGAGGCGGCTTCTCACACCATCCAGAGGATGTATGGCTGCGAAGTGGGGCCGGAGCGGGCGCTTCCCTCGGCGGG
103 G T L R G Y Y N Q S E A G S H T I Q R M Y G C D V G P D G R F L R G
      -CHO-                               -SH-
410 TACCAGCAGCAGCTTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGCGCTCTTGGACCGCGGCGGACATGGCGGCTCAGATCACCCAGCGCAAG
137 Y Q Q D A Y D G K D Y I A L N E D L R S W T A A D M A A Q I T Q R K
512 TGGGAGACGGCCCATGAGGCGGAGCAGTGGAGAGCCTACCTGGAGGGCGAGTGGTGGAGTGGCTCCGAGATACCTGGAGAAAGGGAAGGAGACCGCTGCAG
171 W E T A H E A E Q W R A Y L E G E C V E W L R R Y L E N G K E T L Q
      -SH-
614 CGCACGGACGCCCCCAAGACGCATATGACTCACCACGCTGTCTCTGAACCATGAGGGCAACCCCTGAGGTGCTGGGGCCCTGAGCTTCTACCCCTGGGAGATCACA
205 R T D A P K T H M T H H A V S D H E A T L R C W A L S F Y P A E I T
      -SH-
716 CTCACCTGGCAGCGGATGGGAGGAGCAGACCCAGGACACGGAGCTGCTGGAGACCGGCGCTGACGGGATGGAACTTCCAGAAAGTGGCGCTGTGTGGT
239 L T W Q R D G E D Q T Q D T E L V E T R P A G D G T F Q K W A S V V
818 GTGCCCTTGGACAGGAGCAGAGATACACCTGCCATGTGCAGCATGAGCGCTGCGCCAAAGCCCTCACCCCTGAGATGGGAGCGGTCTCCAGGCCACCATC
273 V P S G Q E Q R Y T C H V Q H E G L P K P L T L R W E P S S Q P T I
      -SH-
920 CCCATCGTGGGCATGATTGCTGGCCCTGCTCTCTTTGGAGCTGTGATCGCTGGACCTGTGGTGGCTGCTGTGATGTGGAGGAGGAAGAGCTCAGATAGAAAA
307 P I V G I I A G L V L F G A V I A G A V V A A V M W R R K S S D R K
1022 GGAGGGACTACTCTCAGGCTGCAAGCAGTGCAGAGTCCCGAGGCTCTGATATGCTCTCACAGCTTGTAAAGTGTGACAGCAGCTGCCCTGTGTGGGACTGA
341 G G S Y S Q A A S S D S A Q G S D M S L T A C K V
      -SH-
1124 GAGGCAAGATTGTCTCCGCCCTTCCCTTTGTGACTTGAAGAACCCCTGACTTTGTTTCTGCAAGGACCTGCAATGTGTGTGTTCTTGTAGGCATAATGT
1226 GAGGAGTGGGAGACCACCCACCCCATGTCCACCATGACCCCTCTGCCACGCTGACCTGTGCTCCCTCCCAATCATCTTTCCTGTCAGAGAGGTGG
1328 GGCTGAGGTGTCTCCATCTCTGCTCAACTTCATGGTGCAGTGTAACTTTTCTCCCTATTAAATTAGAACCTGACATATTC

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FIGURE 3. Sequence of LA45 and comparison with class I- and class I-like proteins. (A) Nucleotide sequence of LA45 cDNA insert and the deduced amino acid sequence (written in single letter code). A putative deletion on position 54 or 55 is marked with an asterisk. (#) Amino acids alanine, asparagine, glycine, or valine. The glycosylation site is indicated by -CHO-. Cysteine residues are depicted (SH). (B) Comparison of LA45 protein sequence with human HLA class I and HLA class I like proteins; a dash indicates identity to LA45. Aw68, A3, A2, B27 are the amino acid sequences of HLA-Aw68, HLA-A3, HLA-A2, and HLA-B27 derived from the SWISS-PROT data bank (code: HA1H\$HUM, HA1C\$HUM, HA1A\$HUM; HA1P\$HUM). HLA-E, JWT15 and RS5 are the non-HLA-A, B, C protein sequences published in references 21, 22 and 23, respectively. These sequence data have been submitted to the EMBC/GenBank Data Libraries and have been assigned accession number X51745.

Besides the discrepancy in the serological reaction pattern between HLA class I proteins and LA45, the two proteins also differ in the association to the β_2m . HLA class I proteins are known to be associated with β_2m , a protein of 12 kD. This protein band was never found to be co-precipitated with LA45 (Fig. 4, lanes *e* and *i*). Therefore, we raised the question whether the LA45 protein represents an unusual, non- β_2m -associated member of the MHC class I family with a cellular distribution pattern (Fig. 1) clearly different from conventional HLA class I proteins, or whether our LA45 antibody is simply unusual and detects a hidden epitope of an otherwise classical HLA-A,B,C molecule that only becomes surface-exposed upon

B

signal peptide

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LA45      MAVMAPRTLVLVLLSGAL/ILTQWA
A3        MARGDQ-----L-----A-----
A2        -----F-----A-----
HLA-E     -VDG--L--S-E-A-----
JTW15     -VDG--L--S-E-A-----

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alpha-1 domain

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LA45      GSHWMRYFFYTSVSRPGRGEPFLIAGVYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYDRNTRNVKAQSQTDRVDLGLRGGYNNQSEA
Aw68     -----S-----
A3        -----S-----F-----
A2        -----S-----F-----
B27      ---S---H-----T-----L-----P-E-----E-QIG--KA--E--R--LR-----
HLA-E    ---SLK--H-----S-----N--P-V--M--S--E--SARDTA-IF--N--R-----
JTW15    ---SLK--H-----S-----N--P-V--M--S--E--SARDTA-IF--N--R-----
RS5      ---SLK-IPL-CPG-A-----S-----E-----N--P-V--M--S--E--SARETAKIF--N-R-V-A---DT

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alpha-2 domain

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LA45      GSHTIQRMYGCDVGPDGRFLRGYQQDAYDGGKDYIALNEDLRSWTAADMAAQITQRKWEAHEAEQWRAYLEGECEVWLRRYLENGKETLQRT
Aw68     ---M---S-----R-----K-----T--KH--A-V-----
A3        ---L---S-----R-----K-----A--L--D-T-----
A2        ---L---S-----W-----H-Y-----K-----T--KH--A-V--L-----
B27      ---L--N--H-----L--H-----S-----T-----A-RV--L-----A
HLA-E    ---L-W-H--EL--R-----E-F-----LT-----V-T--SEQ-SND-S--MQ---DT--HK--K--LHL
JTW15    ---L-W-H--EL--R-----E-F-----LT-----V-T--SEQ-SND-S--MQ---DT--HK--K--LHL
RS5      ---L-W-H--EL--R-----E-F-----LT-----V-T-L-SE--SND-S--DDQ---DT--HK--K--LHL

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alpha-3 domain

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LA45      DAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWRDGEDQTDTELVETRPAGDGTQFKWASVVVPSGQEQRYTCHVQHEGLPKPLTLRW
Aw68     -----L-----A-----
A3        -----L-----A-----
A2        -----L-----A-----
B27      -P---V---PI-----G-----R-E-A-----E-----
HLA-E    EP---V---PI-----G-----Q--GH-----A-----E-----E-V-----
JTW15    EP---V---PI-----G-----Q--GH-----A-----E-----E-V-----
RS5      EP---V---PI-----G-L-----E-Q--GH-----A-----E-----E-V-----

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transmembrane region

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LA45      EPSSQPTPIVGIAGLVLFGLAVVAVVAVVWRRKSS
A3        -L-----L-----T-----
A2        -----T-----
HLA-E    K-----L-S-VS-----I-K---
JTW15    K-----L-S-VS-----I-K---
RS5      RAA-----L-S-VS-----I-K---

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cytoplasmic tail

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LA45      DRKGGSYSQAASSDSAQGSMSLTACKV
A3        -----T-----V-----
A2        -----V-----
HLA-E    GG-----K-EW-----ESHSL
JTW15    GG-----K-EW-----ESHSL
RS5      GG-----K-EG-----ESHSL

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activation. A sequential immunoprecipitation revealed that W6/32 and LA45 antibody and antigen do not crossreact with each other. Furthermore, we could show that cap formation induced by W6/32 or anti- β_2m antibodies did not cap LA45 molecules and LA45 antibody did not induce capping of HLA class I molecules. This might suggest that LA45 is a non- β_2m -associated HLA class I α chain and structurally different from conventional HLA-A,B,C molecules recognized by W6/32 and anti- β_2m . These experiments stress the nonassociation of β_2m to LA45 and argue against a possible weak association as it is described for some mouse class I antigens (20). From all these data LA45 could not be termed a classical HLA class I

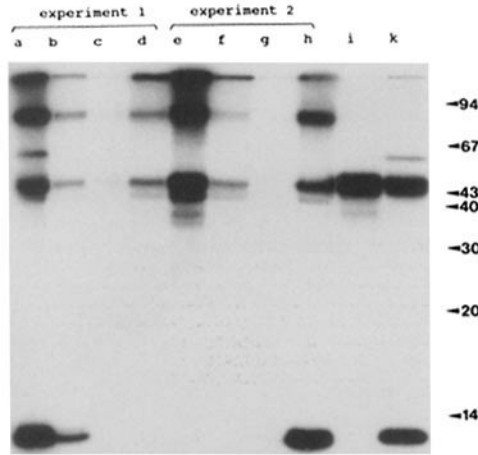


FIGURE 4. Sequential immunoprecipitations of ^{125}I -labeled HUT102 cells (16% SDS-PAGE). The cell lysate was divided into two portions. In the first experiment the cell lysate was cleared with W6/32 (lane *a* shows the first round, lane *b* the second, and lane *c* the fifth round of clearing with W6/32), and afterwards LA45 was precipitated (lane *d*). Preclearing of the same cell lysate with LA45 antibody was done in the second experiment (lane *e*: first cycle, lane *f*: second cycle, and lane *g*: fifth cycle precipitation with anti LA45), and then the lysate was incubated with W6/32, as shown in lane *h*. Lanes *a-h*: precipitated proteins under nonreducing conditions. Lane *i* shows the reduced LA45 precipitate from the first round of the second experiment. Molecular masses shown in kilodaltons.

antigen. Therefore, we assume the expression of structurally altered HLA class I proteins on T lymphocytes upon activation.

In the mouse system the Q_a/TL antigens represent a group of so called class I like proteins. The human equivalent of these Q_a/TL proteins has not yet been found. Some non-HLA-A,B,C class I genes have been cloned (21-23). The cellular expres-

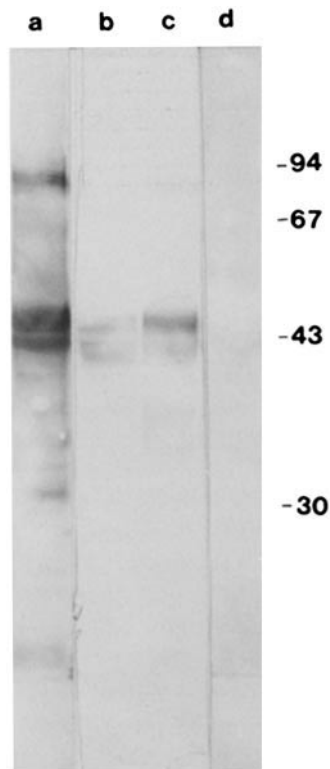


FIGURE 5. Reactivity of LA45 antibody in a Western Blot (12% SDS-PAGE). (Lane *a*) W6/32 precipitated proteins were detected with biotinylated LA45 antibody and extra-avidin alkaline phosphatase; (lane *b*) lysate of resting lymphocytes, (lane *c*) lysate of HUT102 cells; (lane *d*) lysate of the cell line K562. Lysates in lanes *b*, *c*, and *d* were analyzed with LA45 antibody and the Protoblot kit from Promega-Biotech.

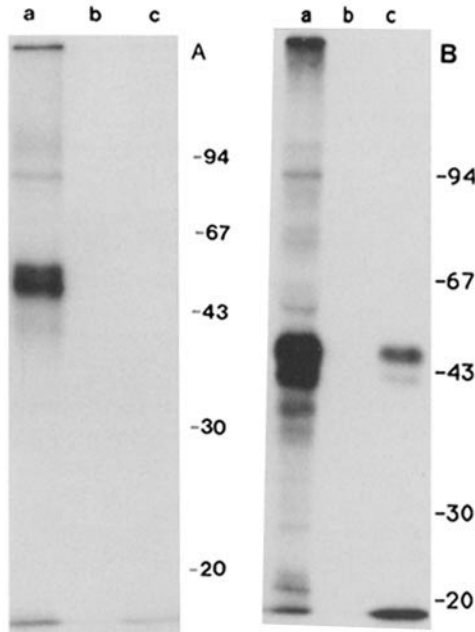


FIGURE 6. (A) Sequential immunoprecipitations of ^{125}I -labeled HUT102 cells (12% SDS-PAGE). The cell lysate of ^{125}I -labeled HUT102 cells was treated for five cycles of 2 h with HC10 immunoabsorbents (lane *a* and *b*: first and fifth round). Lane *c* shows the precipitate of LA45 from the same lysate in the sixth round. (B) Sequential immunoprecipitations of ^{125}I -labeled HUT102 cells (12% SDS-PAGE). Preclearing of the cell lysate with HC10 antibody (lanes *a* and *b*: first and fifth round). Lane *c* shows the precipitate of W6/32 antibody from the same lysate in the sixth round. Molecular masses shown in kilodaltons.

sion pattern of these class I-like clones, as determined by Northern Blotting is, concerning T lymphocytes, similar to that of LA45. When aligning the published sequences, LA45 is only ~75–76% homologous to these genes, compared with a homology of 93% to HLA-A3 (Fig. 3 B). Alloantisera have been described that seem to react with non-HLA-A,B,C class I molecules expressed by activated but not resting lymphocytes (24–27). All so far reported proteins differ from LA45 as they are $\beta_2\text{m}$ associated and have a slightly lower molecular mass than simultaneously studied classical HLA-A,B,C antigens. In contrast, LA45 is not associated to $\beta_2\text{m}$ and has an identical molecular mass to the α chain precipitated by W6/32.

The noncovalent association of the α chain to the $\beta_2\text{m}$ is mainly generated by some amino acids in the α_3 -subunit of the heavy chain in a very short distance to the $\beta_2\text{m}$ in the formed complex (28). We checked the protein sequence of LA45 for mutations in these amino acids and could not detect any different amino acid compared with the HLA-A2 protein sequence. Therefore, the lack of the $\beta_2\text{m}$ association of LA45 cannot be explained by a mutation in the amino acids responsible for the protein aggregation.

The association of the heavy chain and the $\beta_2\text{m}$ occurs posttranslationally in the cytoplasm and is accompanied by a conformational change of the heavy chain (1, 5). This alteration can be reversed by *in vitro* denaturation of HLA class I aggregates. Most of the antibodies against a heavy chain- $\beta_2\text{m}$ complex do not react with the free heavy chains. Furthermore, it was stressed that the association of $\beta_2\text{m}$ to the heavy chain is essential for the membrane transport of functional class I proteins. In contrast, Allen et al. (29) demonstrated that the transfected murine class I molecule H-2D^b can be surface expressed in the absence of $\beta_2\text{m}$. These free heavy chains display a significantly altered conformation unrecognized by allospecific and D^b-

restricted CTL or by most alloantibodies. These findings raised the idea, that our LA45 is a structurally altered class I protein, expressed without β_2m , and therefore is not recognized by W6/32. To delineate this issue, we performed a Western Blot with purified HLA class I proteins denatured *in vitro* by SDS-PAGE. We could detect these antigens with the antibody LA45 on the nitrocellulose. Therefore, denaturation of conventional W6/32-reactive HLA-A,B,C molecules and dissociation from β_2m thus seems to alter α chains in such a way that they become reactive with our LA45 antibody (Fig. 5, lane *a*).

This conclusion is further supported by a sequential immunoprecipitation in which we showed that the LA45 antibody and an antibody HC10, reacting with *in vitro* denatured class I heavy chains, recognize the same proteins on HUT102 cells (Fig. 6 *A*, lanes *a-c*). These molecules are structurally different from conventional, β_2m -linked MHC class I proteins and can only be detected with antibodies recognizing the free heavy chain such as LA45 antibody, but not with antibodies reacting with the intact heavy chain- β_2m complex such as the classical HLA-A,B,C-reactive antibody, W6/32.

This all confirms the previous speculation that activated T cells express structurally altered, non- β_2m -associated heavy chains. Whether these structures represent a separate alloantigen system remains to be seen. However, mitogen-stimulated MNC of 12 normal donors showed LA45 reactivity, although they had mismatching HLA phenotypes with HUT102 cells. Furthermore, in an isoelectrofocusing experiment the LA45 precipitates showed the same reaction pattern when compared with the W6/32 precipitates, although the bands precipitated by LA45 antibody showed a different intensity to W6/32 precipitates, and a certain preference for HLA-A locus proteins were detected (data not shown). This leads us to the conclusion that on activated T cells and possibly other cells, products of all HLA class I gene loci can be found in association with β_2m as well as in a free, conformationally altered form. Additionally, the LA45 antibody defines a monomorphic determinant on free HLA class I heavy chains.

So far, we have not tested if these "free" heavy chains with altered conformation get surface expressed without a biosynthetic association with β_2m . Another possibility, which we in fact favor, would be a functional exchange of a β_2m with a third-party molecule. One candidate for such an associated molecule would certainly be the 38 kD protein observed by us, which was co-precipitated by LA45 antibody in three of five experiments. The trivial possibility that an exchange of human β_2m with constituents and in particular bovine β_2m (30) might be possible, for the *in vitro* changes could be ruled out. Identical results were obtained with culture media supplemented with human serum.

Our working hypothesis for these findings is that activation of T lymphocytes is accompanied by loss of association of β_2m with the known heavy chain HLA class I molecules. A likely explanation for the lack of heavy chain- β_2m association is that the heavy chain is associated with a new structure, thereby displacing β_2m . It seems probable that this change has functional importance given the presence of non- β_2m -associated heavy chains on activated cells but apparently not on precursor cells. The possible nature of new molecules with which the "free" heavy chains can associate and the potential functional importance of such associations are important issues arising from these studies.

Summary

We present here the molecular characterization of a new activation-induced surface structure on human T lymphocytes, termed LA45, with high homology (93% at protein level) to MHC class I molecules. Antigen modulation and sequential immunoprecipitation experiments revealed that LA45 and HLA class I proteins do not crossreact with the corresponding antibodies. Furthermore, LA45 is not associated with β_2 -m. On the other hand, we could show that the separation of HLA-A,B,C and β_2 m molecules, induced by SDS-denaturation, leads to a conformational change in the heavy chain in such a way that it becomes reactive with LA45. The 90/45 kD LA45 proteins thus appear to be non- β_2 m-associated MHC class I α chains that are selectively expressed by activated but not by resting human T lymphocytes.

We thank S. Goyert (New York) for providing us the Okayama-Berg vector pcEXV3, H. L. Ploegh (Amsterdam) for the mAb HC10, W. Schnabl (Vienna) for his help with computer analysis, and A. McMichael (Oxford), as well as F. Bach (Minnesota), for valuable suggestions and critical reading of the manuscript.

Received for publication 17 August 1989 and in revised form 22 January 1990.

References

1. Ploegh, H. L., H. T. Orr, and J. L. Strominger. 1981. Major histocompatibility antigens: the human (HLA-A,B,C) and murine (H-2K, H-2D) class I molecules. *Cell*. 24:287.
2. Due, C., M. Simonsen, and L. Olsson. 1986. The major histocompatibility complex class I heavy chain as a structural subunit of the human cell membrane insulin receptor: implications for the range of biological functions of histocompatibility antigens. *Proc. Natl. Acad. Sci. USA*. 83:6007.
3. Samson, M., J.-L. Cousin, and M. Fehlman. 1986. Cross-linking of insulin receptors to MHC antigens in human B lymphocytes: evidence for selective molecular interactions. *J. Immunol.* 137:2293.
4. Schreiber, A. B., J. Schlessinger, and M. Edidin. 1984. Interaction between major histocompatibility complex antigens and epidermal growth factor receptors on human cells. *J. Cell Biol.* 98:725.
5. Krangel, M. S., H. T. Orr, and J. L. Strominger. 1979. Assembly and maturation of HLA-A and HLA-B antigens in vivo. *Cell*. 18:979.
6. Lancet, D., P. Parham, and J. L. Strominger. 1979. Heavy chain of HLA-A and HLA-B antigens is conformationally labile: a possible role for beta₂microglobulin. *Proc. Natl. Acad. Sci. USA*. 76:3844.
7. Parham, P., C. J. Barnstable, and W. F. Bodmer. 1979. Use of a monoclonal antibody (W6/32) in structural studies of HLA-A,B,C antigens. *J. Immunol.* 123:342.
8. Stam, N. J., H. Spits, and H. L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137:2299.
9. Gubler, U., and B. J. Hoffmann. 1983. A simple and very efficient method for generating cDNA libraries. *Gene (Amst.)*. 25:263.
10. Short, J. M., J. M. Fernandez, J. A. Sorge, and W. Huse. 1988. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res.* 16:7583.
11. Miller, J., and R. N. Germain. 1986. Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. *J. Exp. Med.* 164:1478.

12. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573.
13. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.
14. Lipman, D. J., and W. R. Pearson. 1985. Rapid and sensitive protein similarity search. *Science (Wash. DC).* 227:1435.
15. Holter, W., R. Grunow, H. Stockinger, and W. Knapp. 1986. Recombinant interferon-induced interleukin-2 receptors on human peripheral blood monocytes. *J. Immunol.* 136:2171.
16. Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.).* 227:680.
17. Parham, P., C. E. Lomen, D. A. Lawlor, J. P. Ways, N. Holmes, H. L. Coppin, R. D. Salter, A. M. Wan, and P. D. Ennis. 1988. Nature of polymorphism in HLA-A, -B and -C molecules. *Proc. Natl. Acad. Sci. USA.* 85:4005.
18. Parham, P., D. A. Lawlor, C. Lomen, and P. D. Ennis. 1989. Diversity and diversification of HLA-A,B,C alleles. *J. Immunol.* 142:3937.
19. Robb, R. J., W. C. Greene, and C. M. Rusk. 1984. Low and high affinity cellular receptors for Interleukin 2. *J. Exp. Med.* 160:1126.
20. Beck, J. C., T. H. Hansen, S. E. Cullen, and D. R. Lee. 1986. Slower processing, weaker β_2m -association and lower surface expression of H-2L β are influenced by its amino terminus. *J. Immunol.* 137:916.
21. Koller, B. H., D. E. Geraghty, Y. Shimizu, R. DeMars, and H. T. Orr. 1988. HLA-E: a novel HLA class I gene expressed in resting T lymphocytes. *J. Immunol.* 141:897.
22. Mizuno, S., J. A. Trapani, B. H. Koller, B. Dupont, and S. Y. Yang. 1988. Isolation and nucleotide sequence of a cDNA clone encoding a novel HLA class I gene. *J. Immunol.* 140:4024.
23. Srivastava, R., M. J. Chorney, S. K. Lawrance, J. Pan, Z. Smith, C. L. Smith, and S. M. Weissman. 1987. Structure, expression and molecular mapping of a divergent member of the class I HLA gene family. *Proc. Natl. Acad. Sci. USA.* 84:4224.
24. Fauchet, R., M. Boscher, O. Bouhallier, G. Merdrignac, B. Genetet, P. Turmel, and D. J. Charron. 1986. New Class I in Man: Serological and molecular characterization. *Hum. Immunol.* 17:3.
25. Paul, P., R. Fauchet, M. Y. Boscher, B. Sayagh, M. Masset, G. Merdrignac, J. Dausset, and D. Cohen. 1987. Isolation of a human major histocompatibility complex class I gene encoding a nonubiquitous molecule expressed on activated lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:2872.
26. Gazit, E., Y. Gothelf, R. Gil, S. Orgad, T. B. Pitman, A. L. M. Watson, S. Y. Yang, and E. Y. Yunis. 1984. Alloantibodies to PHA-activated lymphocytes detect human Qa-like antigens. *J. Immunol.* 132:165.
27. Mitsuishi, Y., A. Falkenrodt, A. Urlacher, M. M. Tongio, and S. Mayer. 1986. New human MHC class I antigens segregating with HLA-A antigens detected on some lymphocyte subpopulations. *Hum. Immunol.* 15:175.
28. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (Lond.).* 329:506.
29. Allen, H., J. Fraser, D. Flyer, S. Calvin, and R. Flavell. 1986. Beta₂microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen H-2D^b or of a truncated H-2D^b. *Proc. Natl. Acad. Sci. USA.* 83:7447.
30. Bernabeu, C., M. van de Rijn, P. G. Lerch, and C. P. Terhorst. 1984. b2-Microglobulin from serum associates with MHC class I antigens on the surface of cultured cells. *Nature (Lond.).* 308:642.