either mediated by FcγRII, complement receptors, or both. These divergent roles for FcRs and complement in autoimmune disease offer an explanation for the apparent paradox that deficiencies in complement increase the risk of lupus (*37*, *38*). The complement system may regulate autoreactive B cells (*39*) as well as contribute to IC clearance, whereas FcγRIII mediates the inflammatory activation by ICs. Thus, deficiencies in complement would result in an increase in autoantibodies and a reduction of IC clearance with a corresponding increase in IC deposition, thereby increasing FcR-mediated activation. These studies and ours indicate that complement and Fc receptors have evolved for distinctly different roles in their interaction with ICs. Complement has been shown to be essential for innate immunity against microbial pathogens, requiring natural antibodies to mediate their protective effect (*40*, *42*), whereas FcγRs have emerged as the principal system for coupling antigen-specific IgG antibodies to cellular effector responses and play a minor role in host innate immunity (*29*, *43*, *44*). Therefore, this distinction argues for the development of new therapeutic strategies based on FcR blockade for the treatment of autoimmune glomerulonephritis.

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45. C57Bl/6/129 $\gamma^{-/-}$ mice (*23*) were successively backcrossed to NZB/BINJ and NZW/LacJ (Jackson Laboratories, Bar Harbor, ME) for eight generations before self-mating to generate $\gamma^{-/-}$ and $\gamma^{+/-}$ mice. The eighth generation NZB $\gamma^{-/-}$ males and NZW $\gamma^{+/-}$ females were bred to generate a group of 31 $\gamma^{-/-}$ and 39 $\gamma^{+/-}$ B/W female offspring mice. Mice were genotyped by polymerase chain reaction of tail-tip DNA samples. (Primer sequences: neo: CTCGTGCTTTACGGTATCGCC; gamma 1: ACCCTACTCTACTGTCGACTCAAG; gamma 2:TCACGGCTGGCTATAGCTGCCTT. Annealing temperature was 62°C. Knockout and wild-type amplified products were 260 and 224 base pairs, respectively).
46. Urine was collected at 2-week intervals and read by dipstick (2GP Chemstrip, Boehringer-Mannheim, Indianapolis, IN). We scored 3+ determinations (5 mg/ml) as positive for proteinuria. Using metabolic cages (Fisher, Pittsburgh, PA), we obtained 24-hour urine collections from all mice with 3+ proteinuria. Statistical analysis of proteinuria and survival data were done with the StatSoft software package (Tulsa, OK).
47. For histological analysis, formalin-fixed sections were stained with hematoxylin and eosin. For immunofluorescence studies, acetone-fixed cryosections were stained with (1:1000 diluted) fluorescein isothiocyanate (FITC) goat anti-mouse C3 and IgG [Cappel (ICN), Aurora, OH] and were washed extensively. We used $\gamma^{-/-}$ and $\gamma^{+/+}$ C57Bl/6 kidneys as negative controls (*28*).
48. Diluted serum (1:100) from 6- to 7-month-old B/W $\gamma^{-/-}$ and $\gamma^{+/-}$ mice were added to enzyme-linked immunosorbent assay (ELISA) plates coated with C1q (Sigma, St. Louis, MO) for detection of ICs and to dsDNA-coated plates (United Biotech, Mountain View, CA) for detection of antibodies to chromatin. After washing away unbound serum, the appropriate rat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM (Pharmingen, San Diego, CA) was added. Alkaline-phosphatase–conjugated AKP polyclonal anti-rat IgG (Pharmingen, San Diego, CA) was used as secondary antibody. After incubation with *para*-nitrophenyl phosphate substrate, the samples were read spectrophotometrically at 405 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). Pooled serum from 7-month-old wild-type B/W female mice was used to generate a reference standard curve.

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The Minor Histocompatibility Antigen HA-1: A Diallelic Gene with a Single Amino Acid Polymorphism

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The minor histocompatibility antigen (mHag) HA-1 is the only known mHag for which mismatching is correlated with the development of severe graft versus host disease (GvHD) after human leukocyte antigen–identical bone marrow transplantation. HA-1 was found to be a nonapeptide derived from an allele of the *KIAA0223* gene. The HA-1–negative allelic counterpart encoded by *KIAA0223* had one amino acid difference from HA-1. Family analysis with HA-1 allele-specific polymerase chain reaction showed an exact correlation between this allelic polymorphism and the HA-1 phenotype. HA-1 allele typing of donor and recipient should improve donor selection and allow the determination of bone marrow transplantation recipients with high risk for HA-1–induced GvHD development.

Bone marrow transplantation (BMT) is the current treatment for hematologic malignancies, severe aplastic anemia, and immune deficiency disease. A frequent and life-threatening complication after allogenic human leukocyte antigen (HLA)–identical BMT is GvHD (*1*). Disparities in genes other than the major histocompatibility complex (MHC), referred to as minor histocompatibility antigens (mHags), are clearly involved in the development of GvHD. The mHags are recognized by T

cells and were shown to be peptides derived from intracellular proteins presented by MHC molecules (2–4). A retrospective analysis revealed a significant association between mismatching for the mHag HA-1 and the development of severe GvHD after HLA-identical BMT (5). HA-1 is recognized by HLA-A*0201–restricted cytotoxic T cells (CTLs), is present in 69% of the HLA-A*0201–positive population, and is only expressed by cells of hematopoietic origin (6, 7). Family analysis demonstrated a Mendelian mode of inheritance for HA-1 and segregation independent from the MHC complex (8). To identify the mHag HA-1, HLA-A*0201 molecules were purified from the HA-1–expressing Epstein-Barr virus–transformed B lymphoblastoid (EBV-BL) cell line Rp (9). The HLA-A*0201–bound peptides were isolated and fractionated by multiple rounds of reversed phase high-performance liquid chromatography (HPLC) with different organic modifiers and different gradients (10).

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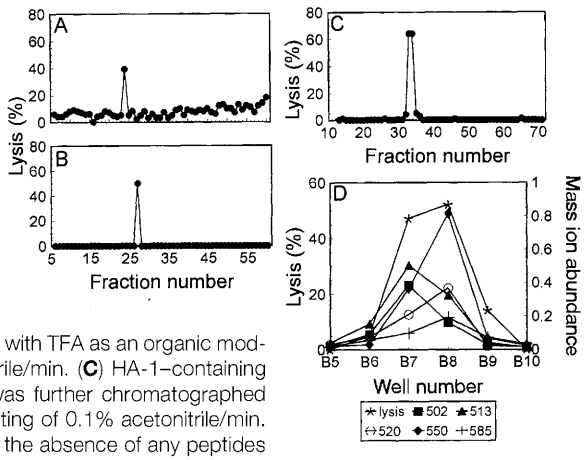
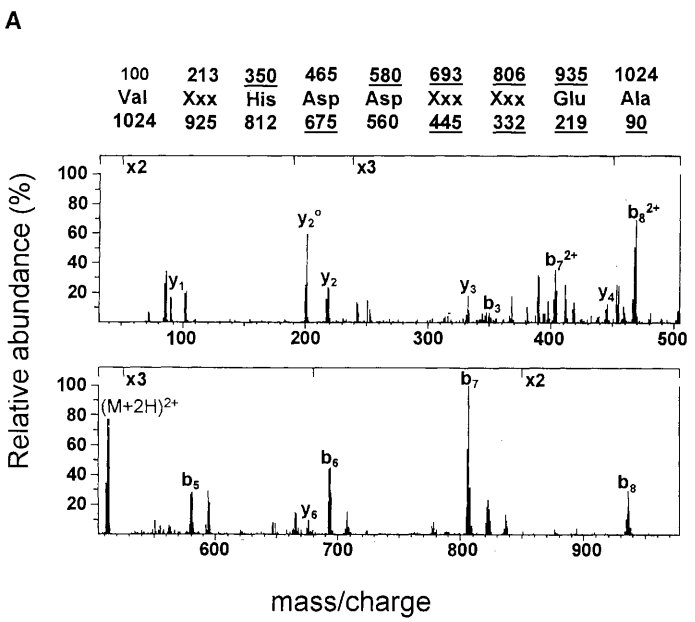


Fig. 1. Reconstitution of HA-1 with HPLC-fractionated peptides eluted from HLA-A*0201 molecules in a ^{51}Cr-release assay with mHag HA-1–specific T cell clone 3HA15. (**A**) Peptides were eluted from 9×10^{10} HA-1– and HLA-A*0201–positive Rp cells and separated by reversed phase HPLC with HFBA as an organic modifier and a gradient of 1% acetonitrile/min. (**B**) Fraction 24 of the first HPLC dimension that contained HA-1 activity was further fractionated by reversed phase HPLC with TFA as an organic modifier and a gradient of 0.7% acetonitrile/min. (**C**) HA-1–containing fraction 27 of the second gradient was further chromatographed with a third shallower gradient consisting of 0.1% acetonitrile/min. Background lysis of T2 by the CTL in the absence of any peptides was in (A) 3%, and in (B) and (C) 0%. Positive control lysis was in (A) 99%, in (B) 74%, and in (C) 66%. (**D**) Determination of candidate HA-1 peptides. HPLC fraction 33 from the separation in (C) was chromatographed with an on-line microcapillary column effluent splitter and analyzed by electrospray ionization mass spectrometry and a ^{51}Cr-release assay. HA-1–reconstituting activity as percent specific release was compared with the abundance of peptide candidates measured as ion current.

The fractions were analyzed for their capacity to reconstitute HA-1–specific lysis of T2 cells, by an HA-1–specific CTL clone, in a ^{51}Cr-release assay (Fig. 1, A to C) (11). The two active fractions (33 and 34) of the third HPLC fractionation were analyzed by microcapillary HPLC–electrospray ionization tandem mass spectrometry (12). Because more than 100 different peptides were present in these fractions, about 40% of fraction 33 was chromatographed with an on-line microcapillary column effluent splitter (2, 13) and simultaneously analyzed by tandem mass spectrometry and ^{51}Cr-release assay (Fig. 1D). Five peptide species [at mass-to-charge (m/z) ratios of 550, 520, 513, 585, and 502] were present in fractions that contained HA-1–reconstituting activity and absent in fractions without activity. To determine which of the five candidates was the HA-1 peptide, we fractionated a second HA-1 purification from the EBV-BL cell line Blk (9) under similar conditions, except that the third dimension separation was done on a microcapillary HPLC column. Mass spectrometric analysis of the peptides in the single peak of reconstituting activity revealed that the only candidate species that was present was that of m/z 513 (14).

The m/z 513 peptide was analyzed by collision-activated dissociation (CAD) analysis and determined to have the sequence VXHDDXXEA (X stands for either Ile or Leu, which cannot be distinguished by mass spectrometry under these conditions) (Fig. 2A) (15). Of all possible isomers containing either Ile or Leu in place of X in this sequence, only synthetic peptide



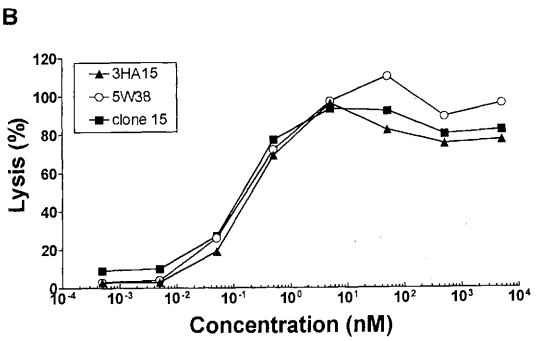


Fig. 2. Sequencing of mHag HA-1 peptide by tandem mass spectrometry. (**A**) CAD mass spectrum of peptide candidate with m/z of 513. (**B**) Reconstitution assay with different concentrations of synthetic mHagHA-1 peptide with three HA-1–specific T cell clones, 3HA15, clone 15, and 5W38. Background lysis of T2 by CTLs in the absence of any peptide was for 3HA15, 4%; for clone 15, 10%; and for 5W38, 2%. Positive control lysis was for 3HA15, 46%; for clone 15, 47%; and for 5W38, 48%.

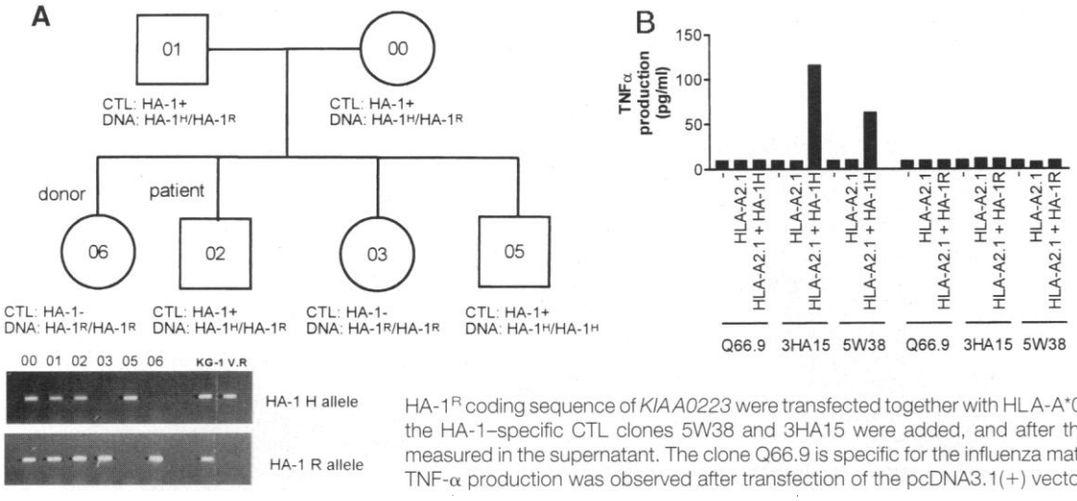


Fig. 3. The *KIAA0223* polymorphism exactly correlated with mHag HA-1 phenotype. (**A**) HA-1 allele–specific PCR reaction in a HA-1–typed family correlated exactly with the HA-1 phenotype. The sizes of the resulting PCR products were consistent with the expected sizes deduced from the cDNA sequence. (**B**) Transfection of the HA-1^H allele of *KIAA0223* leads to recognition by mHag HA-1–specific T cells. The HA-1^H and the HA-1^R coding sequence of *KIAA0223* were transfected together with HLA-A*0201 into HeLa cells. After 3 days the HA-1–specific CTL clones 5W38 and 3HA15 were added, and after the 24 hours TNF-α release was measured in the supernatant. The clone Q66.9 is specific for the influenza matrix protein residues 58 to 66. No TNF-α production was observed after transfection of the pcDNA3.1(+) vector alone.

VLHDDLLEA coeluted with the naturally processed peptide *m/z* 513 on the microcapillary HPLC column (*14*). In a ^{51}Cr-release assay, synthetic peptide VLHDDLLEA was recognized by three different HA-1–specific CTL clones, derived from two unrelated individuals, with a half maximal activity at 150 to 200 pM (Fig. 2B). Thus, the mHag HA-1 is the nonapeptide VLHDDLLEA.

Database searches revealed that the HA-1 peptide VLHDDLLEA was identical at eight of nine residues with the peptide VLRDDLLEA, which is encoded by the partial cDNA sequence designated *KIAA0223* from the acute myelogenous leukemia KG-1 (*16*). Because HA-1 has a population frequency of 69%, we reasoned that the VLRDDLLEA peptide sequence might represent the HA-1–negative allelic counterpart present in the remaining 31% of the population. To test this hypothesis, we analyzed the cDNA sequence spanning this region of *KIAA0223* in EBV-BL cell lines derived from a presumed HA-1 homozygous positive (HA-$1^{+/+}$) individual, from an HA-1 homozygous negative (HA-$1^{-/-}$) individual, and from the KG-1 cell line (*17*). Six of six cDNA sequences from the HA-$1^{-/-}$ individual showed 100% homology with the reported *KIAA0223* sequence. In contrast, six of six sequences amplified from the HA-$1^{+/+}$ individual displayed a two-nucleotide difference from the *KIAA0223* sequence (CTGCA instead of TTGCG), leading to the amino acid sequence VLHDDLLEA. This sequence was identical to that of the peptide identified by mass spectrometry as the HA-1 mHag. The KG-1 cell line expressed both sequences. These results are consistent with the hypothesis that the mHag HA-1 sequence VLHDDLLEA is encoded by an allele (designated HA-1^H) of the cDNA sequence *KIAA0223*. We designated the cDNA sequence of *KIAA0223* present in the data bank as HA-1^R.

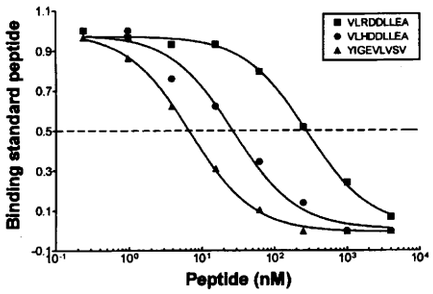


Fig. 4. Binding of HA-1^H and HA-1^R peptides to HLA-A*0201. HA-1^H and HA-1^R peptides were assayed for their ability to inhibit the binding of fluorescent peptide FLPSDCFPSV to recombinant HLA-A*0201 and β_2-microglobulin in a cell-free peptide binding assay. One representative experiment is shown. The IC_{50} was determined on the basis of the results of four experiments and was 30 nM for VLHDDLLEA and 365 nM for VLRDDLLEA.

Further support for this hypothesis was obtained by screening a family consisting of the parents and four children, which had been previously typed for HA-1 with HA-1–specific CTLs, for their *KIAA0223* sequence polymorphism (*18*). All HA-1–negative members of family 1 displayed the HA-1^R sequence, whereas all HA-1–positive members expressed both HA-1^H and HA-1^R sequences. We subsequently designed HA-1 allele–specific polymerase chain reaction (PCR) primers to evaluate a second family previously typed for HA-1 (*19*) (Fig. 3A). The screening of this family also revealed an exact correlation of the HA-1 phenotype and the *KIAA0223* gene polymorphism. Together these results indicate that the *KIAA0223* gene forms a diallelic system of which the HA-1^H allele leads to recognition by the mHag HA-1–specific T cell clones.

To prove that the HA-1^H allele of *KIAA0223* encodes the mHag HA-1, we cloned the appropriate regions of both the HA-1^H and the HA-1^R alleles in eukaryotic expression vectors and transiently transfected them in HA-1–negative HeLa cells in combination with HLA-A*0201 (*20*). The HeLa cells transfected with the HA-1^H sequence, but not the HA-1^R sequence, were recognized by two HA-1–specific CTL clones, as revealed by a tumor necrosis factor-α (TNF-α) release assay (Fig. 3B). The latter absence of recognition was expected, because exogenous VLRDDLLEA peptide was not recognized by HA-1–specific CTL clones 5W38 and clone 15 and was only recognized by 3HA15 at peptide concentrations 10,000 times that necessary for the VLHDDLLEA peptide (*21*). Thus, the mHag HA-1 is encoded by the HA-1^H allele of the *KIAA0223* gene.

Although polymorphism is present at the genetic level, the functional polymorphism is determined by HLA-A*0201–restricted T cell reactivity. Therefore, to determine whether the HA-1^R peptide VLRDDLLEA is presented by HLA-A*0201, we eluted peptides from HLA-A*0201 molecules from a HA-1^R homozygous EBV-BL cell line, fractionated them by reversed phase HPLC, and analyzed them by mass spectrometry. The synthetic VLRDDLLEA peptide was used as a marker. Peptide VLRDDLLEA could not be detected in the HLA-A*0201–eluted peptides (*22*), indicating that this peptide is not presented or is presented in very low amounts by HLA-A*0201 on the cell surface. This is most likely the result of a binding affinity of peptide VLRDDLLEA [50% inhibitory dose (IC_{50}) of 365 nM] that was 1/12 that of peptide VLHDDLLEA (IC_{50} of 30 nM) for HLA-A*0201 (Fig. 4) (*23*). In addition, one amino acid polymorphism can result in different proteasomal processing and differences in peptide presentation (*24*). The absence of the HA-1 ^{R}

peptide in HLA-A*0201 suggests that this allele can be considered as a null allele with regard to HLA-A*0201–restricted T cell reactivity. This further suggests that only BMT from an HA-$1^{R/R}$ donor to an HA-$1^{H/H}$ or HA-$1^{H/R}$ recipient and not the reverse would be significantly associated with GvHD. This was indeed observed in a retrospective study in which HLA-A*0201–positive BMT pairs were typed for HA-1 (*5*). However, it remains possible that other peptides that contain the HA-1^{R} allelic difference bind to other HLA alleles and are recognized by T cells.

Only a few non–sex linked mHag-encoding genes have been identified so far. In mice, two maternally inherited mHags are encoded by two mitochondrial genes with two and four alleles (*25*). Recently, the H13 locus was defined as a H-2 D^{b}–binding nonapeptide differing in one amino acid from its allele. Reciprocal T cell responses could be elicited (*26*). The human mHag HA-2 has only been sequenced on the peptide level (*2*). The identification of the gene encoding the mHag HA-1 is the first example of a human non–sex linked mHag that is derived from a polymorphic gene. The HA-1–encoding *KIAA0223* gene has at least two alleles differing in two nucleotides that lead to a single amino acid difference.

Although the number of different human mHags is probably high, it is envisaged that only few immunodominant mHags can account for the risk for GvHD (*27*). HA-1 demonstrates an immunodominant behavior. First, CTL clones reactive to HA-1 were obtained from peripheral blood lymphocytes of three individuals, each transplanted across a multiple and probably distinct mHag barrier (*6*). Second, in the study mentioned earlier investigating the influence of mHags HA-1 to HA-5 mismatching on the development of GvHD, a mismatch of only HA-1 was significantly associated with GvHD in adult patients (*5*). The immunodominance of HA-1 was recently confirmed by others. Roosnek *et al.* demonstrated HA-1–specific T cell clones in two out of two HA-1 mismatched HLA-identical BMT recipients (*28*). The direct applicability of the identification of the HA-1 alleles is the typing before BMT of HLA-matched donor-recipient combinations. This will improve bone marrow donor selection as well as prediction of development of HA-1–induced GvHD.

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9. The HLA-A*0201–positive EBV-BL cell lines Rp and Blk were derived from peripheral blood mononuclear cells (PBMCs) of, respectively, a BMT and a blood transfusion donor and were cultured in Iscoves modified Dulbecco's medium containing 5% fetal calf serum (FCS). The KG-1 and T2 cell lines were cultured in RPMI-1640 medium containing 3 mM L-glutamine and 10% FCS. The $CD8^{+}$ HLA-A*0201–restricted HA-1–specific CTL clones 3HA15, clone 15, and 5W38 were derived from PBMCs of two patients who had undergone HLA-identical BMT and were cultured as described [(*6*); E. Goulmy, J. W. Gratama, E. Blokland, F. E. Zwaan, J. J. van Rood, *Nature* **302**, 159 (1983)].
10. Peptides were acid eluted from immunoaffinity-purified HLA-A*0201 molecules derived from 9×10^{10} cells from an EBV-BL cell line as described [M. de Bueger *et al.*, *Eur. J. Immunol.* **23**, 614 (1993)]. For both purifications from the EBV-BL cell lines Rp and Blk, peptides were fractionated by several rounds of reversed phase micro-HPLC (Smart System, Pharmacia) as described (*4*), with heptafluorobutyric acid and trifluoroacetic acid (TFA) as organic modifiers and gradients ranging from 1% acetonitrile/min to 0.1% acetonitrile/min. The third dimension microcapillary HPLC fractionation used for the Blk cell line purification consisted of buffer A [0.1% triethylamine (TEA) in H_2O, pH 6.0] and buffer B [0.085% TEA in 60% acetonitrile, pH 6.0]. The gradient was 100% buffer A (0 to 5 min) and 0 to 100% buffer B (5 to 45 min) at a flow rate of 0.5 ml/min.
11. HPLC fractions and synthetic peptides were tested in a ^{51}Cr-release assay as described (*3*, *4*). When HPLC peptide fractions were tested, T2 was incubated with MA2.1 (2 μg/ml) during the ^{51}Cr labeling. Either 1000 or 2500 ^{51}Cr-labeled T2 cells were incubated with samples of the peptide fractions, containing 1/900 to 1/45 of the starting material corresponding to 1×10^{8} cells to 2×10^{9} equivalents, for 30 min at 37°C or 2 hours at room temperature. CTLs were added at effector:target ratios ranging from 9:1 to 33:1.
12. Fractions from third dimension HPLC separation of the Rp purification that contained HA-1 activity were analyzed by microcapillary HPLC–electrospray ionization mass spectrometry as described [(*13*); D. F. Hunt *et al.*, *Science* **255**, 1261 (1992)]. Mass spectra and CAD mass spectra were recorded on a Finnigan-MAT TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer equipped with an electrospray ion source.
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15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; K, Lys; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, either Ile or Leu.
16. GenBank accession number D86976.
17. Total RNA or mRNA was prepared from BLCL with the RNAzol method (Cinaa/Biotecx Laboratories, Houston, TX) or according to manufacturer's instructions (QuickPrep mRNA Purification Kit, Pharmacia Biotech). cDNA was synthesized with 1 mg of RNA as template and with KIAA0223-based reverse primer 5′-GCT-CCT-GCA-TGA-CGC-TCT-GTC-TGCA-3′. To amplify the HA-1 region of KIAA0223 we used the following primers: forward primer 5′-GAC-GTC-GTC-GAG-GAC-ATC-TCC-CATC-3′ and reverse primer 5′-GAA-GGC-CAC-AGC-AAT-CGT-CTC-CAGG-3′. Cycle parameters used were as follows: denaturation at 95°C, 1 min; annealing at 58°C, 1 min; and extension at 72°C, 1 min (25 cycles). The PCR products were purified with the Magic PCR-Preps DNA purification system (Promega) and directly cloned with the pMosBlue T-vector kit (Amersham Life Science). Six independent colonies from each individual were sequenced with the T7 sequencing kit (Pharmacia Biotech).
18. J. Pool, J. M. M. den Haan, E. Goulmy, data not shown.
19. In the case of HA-1 allele–specific PCR amplification, cDNA was synthesized as described (*17*). A PCR amplification was performed with allele-specific forward primers: for the HA-1^{H} allele primer H1, 5′-CCT-TGA-GAA-ACT-TAA-GGA-GTG-TGT-GCT-GCA-3′; for the HA-1^{R} allele primer R1, 5′-CCT-TGA-GAA-ACT-TAA-GGA-GTG-TGT-GTT-GCG-3′. For both reactions the reverse primer as described in (*17*) was used. Cycle parameters used were as follows: denaturation at 95°C, 1 min; annealing at 67°C, 1 min; and extension at 72°C, 1 min (25 cycles).
20. A forward KIAA00223-based PCR primer containing an ATG start codon (5′-CCG-GCA-TGG-ACG-TCG-TCG-AGG-ACA-TCT-CCC-ATC-3′) and a reverse KIAA0223-based PCR primer containing a translational stop signal (5′-CTA-CTT-CAG-GCC-ACA-GCA-ATC-GTC-TCC-AGG-3′) were designed and used in a reverse transcriptase PCR reaction with cDNA derived from a homozygous HA-1^{H} and a homozygous HA-1^{R} BL cell line. Cycle parameters used were as follows: denaturation at 95°C, 1 min; annealing at 60°C, 1 min; and extension at 72°C, 1 min (25 cycles). Purified DNA was directly cloned with the pMosBlue T-vector kit and recloned in the eukaryotic pCDNA3.1(+) vector under the control of a cytomegalovirus (CMV) promoter. We performed transient cotransfections with HLA-A*0201 in HeLa cells using DEAE-Dextran coprecipitation. After 3 days of culture we added HA-1-specific T cells, and after 24 hours we measured the TNF-α release in the supernatant using WEHI cells [C. Traversari *et al.*, *Immunogenetics* **35**, 145 (1992)].
21. J. M. M. den Haan, E. Blokland, C. Reinhardus, E. Goulmy, data not shown.
22. T. L. Bishop, L. M. Meadows, W. Wang, J. Shabanowitz, D. F. Hunt, data not shown.
23. A quantitative assay for HLA-A*0201–binding peptides based on the inhibition of binding of the fluorescent-labeled standard peptide Hbc(18–27), with a substitution of C for F at position 6 (FLPSDCFPSV), to recombinant HLA-A*0201 protein was used [T. H. M. Ottenhoff *et al.*, *J. Immunol. Methods* **200**, 89 (1997); T. L. R. Tan, A. Geluk, M. Toebes, T. H. M. Ottenhoff, J. W. Drijfhout, *ibid.* **205**, 201 (1997)]. In short, various doses of the test peptides were incubated with 1 nM fluorescent standard peptide, HLA-A*0201 yielding about 40 to 60% bound fluorescent standard peptide, and 150 nM β_2-microglobulin for 1 day at room temperature in the dark in a volume of 100 ml. The percent of MHC-bound fluorescence was determined after gel filtration, and the IC_{50} was deduced for each peptide by using one-site competition nonlinear regression analysis with the GraphPad Prism software.
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