Brief report

Naturally processed peptides spanning the HPA-1a polymorphism are efficiently generated and displayed from platelet glycoprotein by HLA-DRB3*0101–positive antigen-presenting cells

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In neonatal alloimmune thrombocytopenia, almost all human platelet antigen (HPA)–1b1b mothers who produce anti–HPA-1a antibody through carrying an HPA-1a fetus are human histocompatibility leukocyte antigen (HLA)–DRB3*0101 positive. It is predicted that the HPA-1a Leu^{33} polymorphism forms part of an HLA-DRB3*0101–restricted T-helper epitope, and acts as an anchor residue for binding this class II molecule. However, it is not known whether any corresponding peptides are naturally processed and presented from platelet glycoprotein. In this study, peptides displayed by a homozygous HLA-DRB3*0101 antigen-presenting cell line were identified after pulsing with recombinant HPA-1a (Leu^{33} plexin-semaphorin-integrin domain). The peptides were eluted from HLA-DR molecules, fractionated by high performance liquid chromatography, and analyzed by tandem mass spectrometry. A "nested set" of naturally presented HPA-1a–derived peptides, each containing the Trp^{25}-Leu^{33} core epitope, was identified, with the most abundant member being the 16-mer Met^{22}-Arg^{37}. These peptides may provide the basis for novel treatments to tolerize the corresponding T-helper response in women at risk of neonatal alloimmune thrombocytopenia. (Blood. 2009;114:1954-1957)

Introduction

Human platelet antigen (HPA)–1a and HPA-1b alloantigens are determined, respectively, by the polymorphism Leu or Pro at position 33 of the β_3 chain (glycoprotein IIIa [GPIIIa]) of the platelet integrin $\alpha_{IIb}\beta_3$.[1] Mismatch between fetal and maternal platelet antigens can lead to the production of maternal immunoglobulin G anti–HPA-1a alloantibodies that cross the placenta and cause thrombocytopenia in an HPA-1a–positive fetus.[2,3] Approximately 2% of white women are HPA-1b homozygous, and 10% of these develop anti–HPA-1a alloantibodies during pregnancy.[2,3] The estimated incidence of severe neonatal alloimmune thrombocytopenia (NAIT) is approximately 1 in 1100 neonates, and the clinical manifestations range from mild purpura to intracranial hemorrhage.[2,3] Maternal responsiveness to HPA-1a shows strong associations with major histocompatibility complex class II, with greater than 95% of responders carrying the DRB3*0101 allele encoding human histocompatibility leukocyte antigen (HLA)-DR52a,[4,5] and up to 94% may carry the DQB1*02 allele.[6] Classical immunoglobulin G antibody responses are dependent on T-helper (Th) cells that recognize antigen as short, processed, linear peptides bound to major histocompatibility complex molecules on antigen-presenting cells (APCs). The identification of the immunodominant helper epitopes on the integrin sequence containing the HPA-1a polymorphic site would be an important step toward understanding and controlling the immune response to HPA-1a. Earlier studies have shown that short, synthetic HPA-1a peptides containing the Leu^{33} polymorphism bind to recombinant DR52a molecules, whereas the Pro^{33} version does not, with Trp^{25} and Leu^{33} predicted to be important anchor residues.[7] The dominant synthetic HPA-1a peptides recognized by peripheral blood Th cells from women alloimmunized with anti–HPA-1a contain the Trp^{25}-Leu^{33} core sequence, with Leu^{33} preferentially at the C-terminus.[8] Recent characterization of the fine specificity of Th cell clones derived from women with NAIT also mapped a putative core epitope spanning Trp^{25}-Leu^{33}.[9] However, antigen processing within APCs is highly selective, with very few peptides typically generated from any given protein for efficient display to Th cells,[10-13] and there is no direct evidence that this core sequence is indeed presented from platelet glycoprotein. The aim of this study was therefore to determine whether DRB3*0101 homozygous APCs, pulsed with HPA-1a, naturally process and present peptides spanning the predicted Trp^{25}-Leu^{33} core epitope.

Methods

Antigen preparation

DNA encoding HPA-1a amino acids 1-62 was cloned into the expression vector pGEX-6P-1, and *Escherichia coli* BL21(DE3) cells were transformed. This sequence corresponds to the complete plexin-semaphorin-integrin (PSI) domain of platelet GPIIIa, which spans the Leu^{33} HPA-1a polymorphism and expresses the HPA-1a antigen.[14] Because the PSI domain is predicted to be on the exterior of the GPIIIa protein[14] and accessible to processing enzymes, we considered this fragment to be representative of the whole protein. The expression of recombinant HPA-1a

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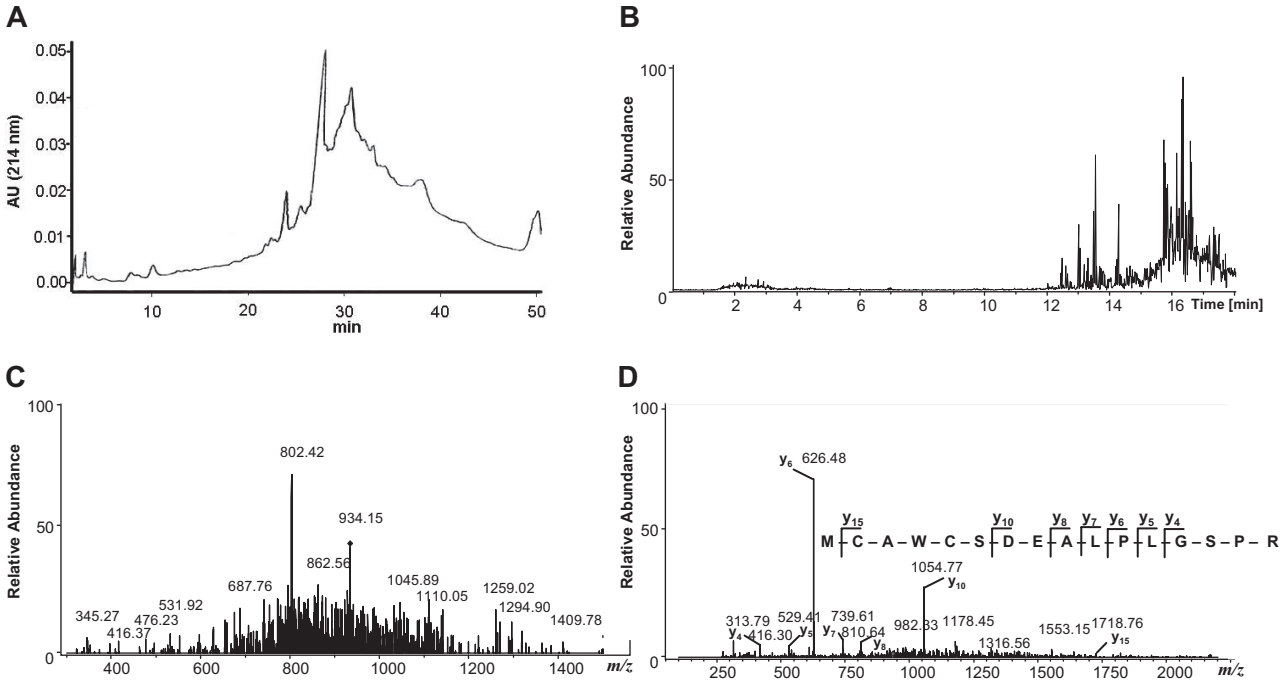

Figure 1. Identification by HPLC and mass spectrometry of HPA-1a–derived peptides eluted from HLA-DR molecules. (A) RP-HPLC profile of the HLA-DR–associated peptides derived from the HLA-DRB3*0101 homozygous B lymphoblastoid cell line, HHKB. Cells were pulsed with glutathione S-transferase–HPA-1a (PSI) domain, and the associated peptides were acid eluted and separated by HPLC using an OD-300, aquapore, C-18, 3-cm × 2.1-mm column. The following gradient conditions were used: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 70% acetonitrile in water containing 0.085% trifluoroacetic acid. The gradient was 0% to 100% B for 50 minutes with a flow rate of 0.5 mL/min. The UV absorbance of the eluates was monitored at 214 nm. (B) Fractions were introduced into the electrospray ionization source of a quadrupole tandem mass spectrometer. The total ion chromatogram of fraction 23 is shown. (C) The survey scan (+MS) for a compound eluting at 16.2 minutes within the *m/z* range of 300-1500 is shown. The most abundant ions were automatically subjected to collision-activated dissociation to yield peptide sequence-specific fragment ions (+MS[2]). (D) The fragmentation spectrum for the doubly protonated precursor ion $[M + 2H]^+$ with an *m/z* of 934.15 was identified as the HPA-1a peptide MCAWCSDEALPLGSPR. The sequence of the peptide was acquired by comparing the observed mass of the type y (amino-terminal cleavage) and type b (carboxyl-terminal cleavage) ions with those of the corresponding theoretical ions, using the Matrix Science web server (www.matrixscience.com).

fused with glutathione S-transferase was induced using isopropyl-β-D-thiogalactopyranoside. The recombinant fused protein was recovered from bacterial lysate and confirmed to bind anti–HPA-1a antibodies, which are sensitive to the conformation of the PSI domain, indicating the integrity of the HPA-1a antigen expression.[15]

APC pulsing with antigen and peptide extraction

HHKB,[16] an Epstein-Barr virus–transformed B-cell line homozygous for HLA-DR B3*0101, was cultured to a concentration of 10^7 cells/mL (total cells 0.7×10^{10}), and then pulsed with 1 mg/mL HPA-1a recombinant antigen. Cells were maintained in RPMI 1640 at 4°C for 2 hours, and then 10 hours at 37°C in 5% CO_2, before washing and lysis in 20 mM Tris-HCl (pH 8)/1% Nonidet P-40/150 mM NaCl/5 mM EDTA (ethylenediaminetetraacetic acid), containing protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin A). Insoluble material was cleared by centrifugation, and the supernatant was filtered (0.45 μM). HLA-DR–peptide complexes were purified from the filtrate by immunoaffinity chromatography using monoclonal anti–HLA-DR antibody (L243) conjugated to protein A-Sepharose. Complexes were eluted from the column with 50 mM diethylamine (pH 11.5) and immediately neutralized in 2 M Tris-HCl (pH 7.4). The HLA-DR–bound peptides were acid eluted at 37°C for 3 hours in 10% acetic acid before ultrafiltration.

High performance liquid chromatography fractionation and mass spectrometry

The peptide pool was separated by capillary reverse-phase (RP)–high performance liquid chromatography (HPLC) using a C-18, 3-cm × 2.1-mm Brownlee cartridge column. The eluted class II-associated peptide fractions were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics), coupled to an UltiMate 3000 LC System (Dionex). Peptide peaks were detected and deconvoluted automatically using Data Analysis software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input to Mascot tandem mass spectrometry (MS/MS) Ions searches of the National Center for Biotechnology Information database.

Results and discussion

Direct biochemical methods were used to identity any peptides processed and displayed from the HPA-1a antigen by APCs expressing the HLA-DRB3*0101 susceptibility allele. An HLA-DRB3*0101 homozygous B-cell line was pulsed with the recombinant HPA-1a PSI domain as antigen, the HLA-DR class II/peptide complexes were recovered, and the peptides were eluted for analysis. RP-HPLC of the peptide pool revealed, as expected, a heterogeneous mixture of sequences (Figure 1A), and 45 fractions were collected. Matrix-assisted laser desorption ionization–time of flight mass spectrometry demonstrated that the most abundant peptides had individual molecular masses of 1502-1831 Da, reflecting typical lengths of 13-15 amino acids. Further characterization using a quadrupole ion trap, equipped with an electrospray ion source, enabled sequence determination of individual peptides.

In fractions 21-23, HPA-1a–derived sequences scored highest in the Mascot search, among those peptides recovered. The profile of these abundant sequences identified a "nested set" of 4 naturally processed and presented HPA-1a peptides (Table 1). The sequences were up to 17 amino acids in length and spanned the Leu[33] polymorphism. Each peptide contained the core residues of the

Table 1. Nested set of glutathione S-transferase–HPA-1a–derived peptides

Peptide	Fragment	Sequence	Length	Observed $[M + 2H]^{2+}$ ions	Calculated* mass $[M^+]$	Theoretical mass $[M^+]$
1	21-37	PMCAWCSDEALPLGSPR	17	916.2	1831.8	1830.5
2	22-37	MCAWCSDEALPLGSPR	16	934.1	1864.7	1866.2
3	24-37	AWCSDEALPLGSPR	14	751.4	1500.7	1500.8
4	25-37	WCSDEALPLGSPR	13	715.9	1429.6	1429.7

LC-MS/MS data were submitted to a Mascot search to identify proteins in the original sample. Four peptides derived from the recombinant HPA-1a antigen were identified in different fractions. All are presumed recovered from the HLA-DRB3*0101 molecules present on the HHKB cells.

*Calculated mass = $([M + 2H]^{2+} \times 2) - 2$.

predicted dominant Th-cell epitope (Trp^{25}-Leu^{33}), and although heterogeneous at the N-terminal, all terminated at Arg^{37}. MS/MS analysis of the most abundant HPA-1a–derived peptide, a 16-mer with an *m/z* value of 934.15^{2+}, is shown in Figure 1B through D.

There have been few other successful studies to identify the class II–associated peptides that are naturally processed from specific antigens, reflecting the technical difficulty in characterizing the complex peptide pools displayed by APCs. Other examples include the classic foreign antigens, hen egg lysozyme[10,17] and tetanus toxoid[11]; the human autoantigens of the α-chain of type IV collagen[12]; and GAD65[13]/insulin[18] targeted, respectively, in Goodpasture's disease and type I diabetes. The current work is the first to report the sequences of naturally processed and presented peptides from a human alloantigen. The dominance of the HPA-1a–derived peptides, which were the most abundant sequences eluted from the APCs, is particularly striking, and will reflect both a high efficiency of processing and the predicted high affinity of the core epitope Trp^{25}-Leu^{33} for the DR52a class II molecule expressing HLA-DRB3*0101. It is increasingly recognized that high affinity for class II alone does not ensure presentation of peptides, and that processing constraints play a key role in epitope selection.[12,13]

The HPA-1a peptides shown to be presented in this study form a "nested set" around the core epitope, a feature typical of the naturally processed peptides derived from other antigens.[12,13] This feature arises when class II molecules bind and protect a corresponding core sequence while this is still part of relatively large antigen fragments at an early stage of processing.[19] Subsequent trimming of the fragments is variable, producing a characteristic set of peptides with different lengths spanning the core epitope.[20,21]

T-cell stimulation[8] and peptide-binding[22] approaches with synthetic peptides have previously been used to map Th epitope(s) of HPA-1, and the polymeric residue Leu^{33} of integrin β_3 was predicted to be a major anchor residue in the putative DRB3*0101 (DR52a) peptide-binding motif. In this study, we demonstrate for the first time that the predominant naturally processed and presented HPA-1a peptides span this predicted epitope. The efficient processing and presentation of the peptides are likely to be an important contributory factor in the immunogenicity of HPA-1a. Such peptides may also provide the basis for novel treatments to tolerize the corresponding Th response in HPA-1b1b women at risk of NAIT with an HPA-1a–positive fetus.

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Authorship

Contribution: G.A.S. performed experiments, discussed the data, and wrote the manuscript; and M.M., R.N.B., and S.J.U. designed the study, discussed the data, and wrote the manuscript.

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