The association of ERAP1 with ankylosing spondylitis (AS) among HLA-B27-positive individuals suggests that ERAP1 polymorphism may affect pathogenesis by altering peptide-dependent features of the HLA-B27 molecule. Comparisons of HLA-B*27:04-bound peptidomes from cells expressing different natural variants of ERAP1 revealed significant differences in the size, length, and amount of many ligands, as well as in HLA-B27 stability. Peptide analyses suggested that the mechanism of ERAP1/HLA-B27 interaction is a variant-dependent alteration in the balance between epitope generation and destruction determined by the susceptibility of N-terminal flanking and P1 residues to trimming. ERAP1 polymorphism associated with AS susceptibility ensured efficient peptide trimming and high HLA-B27 stability. Protective polymorphism resulted in diminished ERAP1 activity, less efficient trimming, suboptimal HLA-B27 peptidomes, and decreased molecular stability. This study demonstrates that natural ERAP1 polymorphism affects HLA-B27 antigen presentation and stability in vivo and proposes a mechanism for the interaction between these molecules in AS. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.019588, 1416–1429, 2012.
cavity close to the catalytic site, as well as four domains; the conformational rearrangement between an open and a closed conformation, presumably induced upon substrate binding, regulates its enzymatic activity (19, 20). The polymorphic residues found among natural ERAP1 variants (21), and often co-occurring in complex allotypes, are located in various topological regions, including some in close proximity to the catalytic site, the substrate binding cavity, or domain junctions. Therefore, they might alter ERAP1 activity by directly affecting catalysis, altering substrate binding, or modulating domain rearrangements. The association of ERAP1 with AS does not by itself reveal the specific feature(s) determining the pathogenetic role of HLA-B27. Indeed, ERAP1 might influence the generation of specific pathogenetic epitopes; have a general effect on the HLA-B27 peptidome, altering the stability or other features of the molecule; or both. This study investigated general effects of ERAP1 polymorphism on the HLA-B27 peptidome by comparing the size distribution, molecular features, and N-terminal flanking sequences of peptides from human cells expressing the AS-associated B*27:04 subtype and different natural variants of ERAP1.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—The following B*27:04-positive cell lines were used: JSL (HLA-A*11; B*27:04, *48; C*02), WEWAK1 (WE-I: HLA-A*11, *24; B*27:04, *62; C*02, *04), and KNE (HLA-A*01, *02:04; B*27:04, *62, *68; C*02, *04). The cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FBS (Invitrogen, Paisley, UK). ME1 (IgG1), an anti-HLA-B7/B27/B22 monoclonal antibody (mAb) that recognizes HC/β2m/peptide complexes (24), was used for immunopurification of HLA-B27.

**Typing of Nonsynonymous SNPs in the ERAP1 and ERAP2 Genes**—DNA purification was performed using the High Pure PCR Template Preparation system (Roche Diagnostics, Barcelona, Spain) following the instructions of the manufacturer. Aliquots of 10 ng were added onto 384-well plates in duplicate, dried, and amplified using specific oligonucleotides for eight non-synonymous SNPs located in the coding sequence of the ERAP1 gene: rs266653, rs266618, rs278955, rs27044, rs30187, rs10050860, rs17482078, and rs2287987 (Table I). Samples were run in an HT7900 Fast Real-Time PCR System and genotyped using SDS2.2 software (both from Applied Biosystems, Invitrogen, Carlsbad, CA) for allelic discrimination. The nonsynonymous SNP rs2549782 (G/T), encoding for the K392N change in ERAP2, was typed by same procedure.

**Sequencing of ERAP1 Variants**—Exons 2–20, encompassing the coding region of ERAP1, were separately amplified via PCR and cloned into M13 for sequencing. PCR products were generated using AmpITaq Gold PCR Master Mix (Applied Biosystems) following standard procedures, purified using ExoSap (USB Corp., Cleveland, OH), and sequenced in a 3730XL instrument (Applied Biosystems). Sequencing primers were either M13-complementary oligonucleotides, for amplicons that included this extension along the ERAP1 sequence, or the specific primers themselves when the amplicons lacked M13-derived sequences. Both strands of amplicons were routinely sequenced.

**Quantitative RT-PCR**—Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Madrid, Spain), and this was followed by digestion with DNase I (Invitrogen, Karlsruhe, Germany). Complementary DNA was synthesized from 250 ng of total RNA using the High Capacity cDNA reverse transcription kit (Invitrogen) according to the manufacturer’s instructions. The primers used for the amplification were purchased from Applied Biosystems. Comparative quantification of gene expression was performed via quantitative RT-PCR with an AB7900HT instrument (Applied Biosystems) using TaqMan probes and Gene Expression Master Mix (Applied Biosystems). Amplifications were carried out with an initial hold at 50 °C for 2 min followed by denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s. The results were expressed as relative mRNA expression quantified with the RQ Manager software and normalized to glyceraldehyde-3-phosphate dehydrogenase transcript levels.

**Western Blot**—About 2 × 10⁶ cells were lysed in 0.5% Igepal CA-630 (Sigma-Aldrich, St Louis, MO), 50 mM Tris HCl, 5 mM MgCl₂, pH 7.4, containing protease inhibitors (Complete Mini (Roche, Mannheim, Germany). After SDS-PAGE (10% slab gels) of whole lysates under reducing conditions, the separated components were electroblotted onto a nitrocellulose membrane (Amersham Biosciences Hybond-ECL) (GE Healthcare, Buckinghamshire, UK) at 20 V overnight using 30% methanol in 50 mM Tris/Gly buffer, pH 8.8, 0.04% SDS. ERAP1, ERAP2, and γ-tubulin, used as an internal standard, were revealed with the 6H9 mAb (a kind gift from Peter van Endert, INSERM, Paris, France), 3F5 (R&D Systems, Minneapolis, MN), or GTU88 (Sigma-Aldrich), respectively, using peroxidase-conjugated goat anti-mouse Ig polyclonal antibody (DakoCytomation, Glostrup, Denmark). The scanned autoradiograms were quantified using TINA 2.09e image analyzer software (Raytest Isotopenmessgeräte, Straubenhardt, Germany).

**Isolation of HLA-B27-bound Peptides**—This was carried out as described elsewhere (25). Briefly, cells were lysed in 1% Igepal CA-630 with a mixture of protease inhibitors (Roche). The soluble fraction was subjected to affinity chromatography using the ME1 mAb. HLA-B27-bound peptides were eluted with 0.1% aqueous TFA at room temperature, filtered through Centricron 3 (Amicon, Beverly, MA) or Vivaspins 2 Hydrostart (VS02H11, Sartorius Stedim Biotech, Göttingen, Germany), concentrated, and subjected to HPLC fractionation in a Waters Alliance system (Waters, Milford, MA) using a Vydac 218TP52-C18 column (Vydac, Hesperia, CA) at a flow rate of 100 μl/min, as described elsewhere (26). Fractions of 50 μl were collected and stored at −20 °C.

**Mass Spectrometry**—Individual HPLC fractions were analyzed via MALDI-TOF MS using a 4800 Proteomics Analyzer (Applied Biosystems), as described elsewhere (27). The mass spectra were acquired in reflector positive mode at 25 kV in the m/z range of 800–2000, using a signal-to-noise ratio (s/n) cutoff of 3, and processed using Data Explorer software, version 4.9 (Applied Biosystems). Sample handling and acquisition parameters were tightly controlled to minimize differences in the experimental conditions among samples to be compared in each experiment.

**Peptide sequencing** was carried out with MALDI-TOF/TOF MS/MS, as described elsewhere (27). Interpretation of the MS/MS spectra was assisted by various tools. Manual inspection of the spectrum usually allowed us to derive a tentative sequence. This was used to screen the human proteome in the human protein entries of the Uniprot/Swiss-prot database (Release 57.6, June 28, 2009, with 20,331 entries), using a window of 0.5 m/z units for both precursor and fragment ions, for a possible match using the Mascot server software. For those sequences showing the highest scores in this preliminary search, the MS-product tool (version 5.9.4) (University of California, San Francisco, CA) was used to match the candidate sequences to our MS/MS spectra.

**Trimming Susceptibility of N-terminal Flanking and P1 Residues**—The susceptibility of flanking (P-2, P-1) and P1 residues to ERAP1...
**ERAP1 and the HLA-B27 Peptidome**

**Table I**

ERAP1 polymorphism in B*27:04 positive cell lines

| SNP       | Position N. | Odds ratio (Ref.) | Consensus | JSL | C1R | KNE | WE-I |
|-----------|-------------|-------------------|-----------|-----|-----|-----|------|
| rs26653   | 380/127     | 1.3 (21)          | g/R       | g/P | g/R | g/R | c/P  |
| rs26618   | 828/276     | 0.99 (21)         | a/I       | a/I | a/I | a/I | a/I  |
| rs27895   | 1037/346    | 1.07 (21)         | g/G       | g/M | g/M | g/M | g/M  |
| rs2287987 | 1045/349    | 0.71 (56)         | a/M       | a/M | a/M | a/M | a/M  |
| rs30187   | 1585/528    | 1.4 (56)          | g/R       | g/R | g/R | g/R | g/R  |
| rs10050860| 1723/575    | 0.71 (56)         | g/D       | g/D | g/D | g/D | a/N  |
| rs17482078| 2174/725    | 0.7 (56)          | g/R       | g/R | g/R | g/R | g/R  |
| rs27044   | 2188/730    | 1.4 (56)          | c/O       | c/O | c/O | c/O | c/O  |

* Only nonsynonymous changes in the coding strands of ERAP1 are shown. Nucleotide and amino acid residue numbering and consensus sequence are from Human ERAP1 Isoform 2 (Accession No.: Q9NZ08-2). Deviations from the consensus sequence are in boldface. Polymorphisms associated with increased risk for AS are underlined. All polymorphic positions were determined via SNP typing and confirmed by genomic sequencing.

**RESULTS**

**ERAP1 and ERAP2 Polymorphism and Expression in HLA-B*27:04-positive Cell Lines**—Four B*27:04-positive cell lines, including 3 LCL and C1R transfectants, were selected on the basis of their expression of eight SNPs encoding nonsynonymous substitutions in ERAP1, including 6 AS-associated ones (9, 21). Sequencing of all of the exons of this gene in the four cell lines confirmed these polymorphisms (Table I). Whereas the coding sequence of ERAP1 from JSL was identical to the consensus and carried all six polymorphisms associated with increased susceptibility to AS, that from WE-I showed six nonsynonymous changes, including all those associated with protection from AS. ERAP1 from C1R04 showed four nonsynonymous changes relative to the consensus. KNE was heterozygous for rs26653, rs26618, and rs27895. This variability allowed us to examine the effect of various combinations of amino acid changes in natural ERAP1 variants on the HLA-B27-bound peptidome. Genotyping of the four cell lines for the nonsynonymous rs2549782 SNP of ERAP2, coding for the K392N change, revealed that WE1, C1R04, and KNE were heterozygous, whereas JSL was homozygous (T/T), expressing only the N392 allele.

Western blot analyses of WE-I, C1R04, and KNE (Figs. 1A and 1B) revealed that these cell lines expressed fairly similar ERAP1 protein levels: the WE-I/C1R04 ratio was 1:1.2, and the C1R04/KNE ratio was 1:1.4 (Fig. 1C, in agreement with previous studies on LCL (31). Although protein levels could not be assessed for JSL because of the loss of this cell line in the course of this study, the relative mRNA expression of ERAP1 was determined for all four cell lines (Fig. 1C). This reflected closely ERAP1 protein levels in C1R04 and WE-I (WE-I/C1R04 ratio, 1:1.3). mRNA expression for KNE was similar to that for WE-I and somewhat lower than that for C1R (C1R:KNE ratio, 1:0.75). JSL showed the lowest mRNA levels (about 0.6:1 and 0.8:1 relative to C1R04 and to WE1 or KNE, respectively). ERAP2 protein expression was similar in C1R04 and KNE and about 2-fold higher in WE-I (supplemental Fig. S1).

**Automated Comparison of HLA-B27-bound Peptide Repertoires**—B*27:04-bound peptide pools were fractionated via HPLC, and each fraction was analyzed using MALDI-TOF MS. Three pairwise comparisons—WE-I/JSL, WE-I/C1R04, and C1R04/KNE—were carried out, following a strategy previously used for HLA-B27 subtype-bound peptidomes (32–34). In order to assess the inherent experimental error in these comparisons, the B*27:04-bound peptides from two independent batches of C1R04 cells, C1R04-I and -II, were separately processed and compared in the same way. For each cell line pair, the MS spectra of correlative HPLC fractions from consecutive chromatographic runs performed under identical conditions were automatically compared using a newly developed software, MShandler (Fig. 2). All the ion peaks in the m/z range of 800–2000 and s/n > 3 were collected from the MS spectra of all HPLC fractions from each...
peptide pool and identified by their $m/z$ and HPLC fraction numbers. Prior to each comparison, a series of filters were applied to remove irrelevant signals and maximize the selection of B27-related ion peaks. The filtered ion peak sets from the two cell lines in each experiment were compared: ion peaks with identical ($|m/z| < 0.2$) in the correlative $m/z$ of the other cell line were considered as shared peptides in the two cell lines and subjected to further analysis. All other peaks were disregarded (supplemental Table S2).

Relative Expression of B*27:04 Ligands in Distinct ERAP1 Contexts—We reasoned that if there were not a general influence of ERAP1 polymorphism on the HLA-B27 peptidome, or in the context of similar ERAP1 molecules, the size distribution of shared peptides in two cell lines would be essentially identical independent of their relative abundance in each cell line. Conversely, if ERAP1 polymorphism has a general influence on peptide trimming, the size distribution of shared B27 ligands will be shifted toward higher molecular mass (Mw) values in the cell line with the less efficient ERAP1 variant, to an extent that would depend on the relative abundance of the peptides compared. Thus, we calculated the intensity ratio (IR) between shared ion peaks in the WE-I/JSL, WE-I/C1R04, C1R04/KNE, and C1R04-I/II comparisons as an estimation of relative peptide abundance. The peptides showing IR > 1 were classified in three subsets: those showing >3-fold, >1.5- to 3-fold, and >1- to 1.5-fold intensity in one relative to the other cell line (supplemental Table S3). We next compared the mean Mw (Fig. 3) and the Mw distribution (Fig. 4) within equivalent subsets in both cell lines. The results are described below.

WE-I/JSL—The shared ion peaks showed a Gaussian size distribution with a mean $m/z$ of 1162.0 (Fig. 4A). For peptides with IR > 1 in each cell line there was a shift of the Gaussian curve from WE-I, relative to JSL, toward higher Mw values.
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Fig. 3. Relative expression and mean Mw of shared B*27:04 ligands in various ERAP1 contexts. For each of the four indicated pairwise comparisons, the shared ion peaks were classified on the basis of their intensity ratio (IR) between both cell lines as indicated. The “IR > 1” set in each cell line includes the three other indicated subsets. The mean m/z value, which in MALDI-TOF MS is equivalent to the mean Mw (M + H⁺), of the ion peaks in each peptide subset was calculated, and the difference (Δm/z) between equivalent subsets from the two cell lines compared was represented for the four comparisons. Data for WE-I/JSL and C1R04 (I/II) are from a single experiment; those for WE-I/C1R04 and C1R04/KNE are the mean ± S.D. of two and three experiments, respectively.

(Δm/z = 33.8). When the different IR subsets were compared, the magnitude of the shift was found to be highest between the IR > 3 subsets (Δm/z = 73.1), somewhat smaller between those with IR > 1.5 to 3 (Δm/z = 49.2), and virtually overlapping (Δm/z = 5.9) in the >1- to 1.5-fold subsets. This comparison was carried out only once because of the loss of JSL in the course of this study.

WE-I/C1R04—The shared ion peaks in these two cell lines showed a Gaussian size distribution with mean m/z values of 1154.3 and 1157.2 in two independent comparisons (supplemental Table S3) (Fig. 4B). The curve of the peptides with IR > 1 in WE-I was shifted toward higher Mw, relative to C1R04, similarly as for JSL (Δm/z = 28.5 ± 3.2). When IR subsets were compared, a curve shift similar to that of WE-I/JSL was observed for the IR > 3 and >1.5 to 3 subsets, although the mean Mw differences were somewhat smaller (Δm/z = 53.5 ± 7.2 and 40.8 ± 3.3, respectively) (Fig. 3) (supplemental Table S3). These results indicate that B27 ligands with high Mw tend to be more abundant in WE-I than in JSL or C1R04, and the opposite is true for ligands with low Mw, but differences are smaller with C1R04 than with JSL.

C1R04/KNE—The ERAP1 molecules in these two cell lines were identical except at positions 127, 276, and 346, where KNE, but not C1R, showed heterozygosity (Table I) (Fig. 4C). This comparison was carried out three times using three independent batches of C1R04 cells and two batches of KNE. The size distribution and average Mw of shared B27 ligands showed significantly smaller differences between both cell lines than in the previous comparisons (Figs. 3 and 4), indicating little effect of the ERAP1 or other cellular background differences between C1R04 and KNE on these features.

C1R04 III—The intensity of individual ion peaks corresponding to B*27:04 ligands showed variability in the two preparations that allowed their classification into IR subsets similar to those observed between different cell lines (supplemental Table S3). To some extent, this might be due to the fact that C1R04-I and -II were grown and processed as independent batches, but it also suggests that, on an individual basis, the ion peak intensity in MALDI-TOF MS might not be a reliable indicator of peptide abundance. Yet, in contrast to previous comparisons, the average Mw of B27 ligands was very similar in both peptide preparations for all IR subsets (Fig. 3), and the corresponding size and length distributions were virtually overlapping in all cases (supplemental Fig. S2), indicating that the size distribution differences observed in other comparisons are not due to the nonquantitative character of ion peak intensity as an indicator of peptide abundance. The value of Δm/z = 9.1 Da observed only between the IR > 3 subsets (Fig. 3) reflects the level of experimental error in this type of comparison when applied globally to large peptide sets.

The results indicate that allelic ERAP1 polymorphism has a global influence on the B*27:04 peptidome, affecting the relative expression of many B*27:04 ligands as a function of peptide size. The observed effects are consistent with the following ERAP1 activity ranking: JSL > C1R04/KNE > WE-I.

Relationship between Size and Length Distribution of B*27:04 Ligands—Because of the wide Mw range among peptides of the same length, translating the observed peptide size into length differences that best reflect the relative activity among ERAP1 variants was not straightforward. Thus, the amino acid sequence of 372 B*27:04 ligands showing various degrees of differential expression among cell lines was determined (supplemental Table S4), and the percentage of peptides of any given length within each Mw range was calculated (Table II). As these sequences were obtained due only to the high intensity of the corresponding ion peaks in the MS spectra and were not preselected otherwise, they were
assumed to represent a fair sampling of the B27 peptidome. Thus, the relationship between Mw and residue length among these peptides was used to translate the Mw differences described in the preceding paragraph into peptide length differences (Fig. 5).

WE-I/JSL—The shared B*27:04 ligands between these cell lines were estimated to include 2.5% 8-mers or smaller peptides, 70.7% 9-mers, 21.1% 10-mers, and 5.8% longer peptides (Fig. 5A). Peptides with IR > 1 showed a skewing against short peptides (9-mers and shorter) and toward long ones (10-mers and longer) in WE-I; the magnitude of the skewing depended on the expression relative to JSL and was highest for the IR > 3 subsets, intermediate for IR > 1.5 to 3 subsets, and low among peptides with IR > 1 to 1.5.

WE-I/C1R04—As in the preceding comparison, peptides with IR > 1 showed a moderate skewing against short peptides and toward long ones in WE-I (Fig. 5B). Length skewing again was more prominent between the IR > 3 subsets, decreased in the IR > 1.5 to 3 subsets, and was marginal in the IR > 1 to 1.5 subsets. The pattern was similar to that in WE-I/JSL, but the differences were smaller.

C1R04/KNE—Length differences among the B*27:04 ligands of these cell lines were noticeable only among the peptide subsets with IR > 3 (C1R04/KNE ratio 0.8 ± 0.0 and

![Diagram](image-url)
ERAP1 and the HLA-B27 Peptidome

The N-terminal Flanking and P1 Residues Determine the ERAP1-dependent Expression of B*27:04 Ligands—Amino acid residues differ widely in their susceptibility to ERAP1 trimming. Susceptible residues in the N-terminal flanking sequences favor the generation of B*27:04 ligands, whereas susceptible P1 residues might favor their destruction. Thus, the N-terminal flanking residues of numerous B*27:04 ligands showing distinct relative expression among cell lines were assigned from the sequence of their parental proteins (supplemental Table S4). The sequenced peptides in each pairwise comparison were classified in those with IR > 3 and those with IR = 1 to 3 in one cell line relative to the other. The flanking (P-2 and P-1) and P1 residues of each peptide were assigned a trimming susceptibility score (supplemental Table S1) based on a previously reported assay (28). For each peptide subset, a mean score was determined for P-2, P-1, P-2 + P-1, and P1. The ratio between the mean scores of equivalent peptide subsets in the cell lines compared was used as a global estimation of their relative susceptibility to trimming. The following observations emerged from this analysis (Table III).

**WE-I/JSL**—Both the flanking and P1 residues of the peptides in the IR > 3 subsets were more susceptible to trimming in WE-I than in JSL (added P-2 + P-1 score ratio = 1.4; P1 score ratio = 2.5), but those in the IR < 3 subsets showed smaller differences. These results strongly suggest that the predominant of B*27:04 ligands in these cell lines is determined by higher or lower susceptibility of the flanking and P1 residues to trimming in the context of the less (WE-I) or more (JSL) active ERAP1 variant, respectively.

**WE-II/C1R04**—The flanking residues of the peptides in the IR > 3 subsets were more susceptible to trimming in WE-I than in C1R04 (added P-2 + P-1 score ratio of 1.4). In contrast, the average susceptibility of P1 residues to trimming was very similar in both cell lines for all peptide subsets, regardless of their relative expression (Table III). Thus, again, the predominance of B*27:04 ligands in these cell lines is correlated with higher susceptibility of the flanking residues to trimming in the context of the less active variant (WE-I). The similar P1 susceptibility suggests that differential epitope destruction contributes less to peptide predominance between these two cell lines. Yet, similarly susceptible P1 residues should be more efficiently trimmed by the most active ERAP1 variant.

**C1R04/KNE**—This analysis revealed smaller differences in the trimming susceptibility of the flanking and P1 residues between the peptides predominant in either cell line than in the previous comparisons (Table III). Score ratios for any given position or peptide subset were no higher than 1.3, and their magnitude did not correlate with IR. Thus, peptide expression differences between C1R04 and KNE seem largely unrelated to ERAP1, in agreement with the similar activity of this enzyme in both cell lines suggested by the size and length distribution of their HLA-B27 peptidomes.

The Internal Sequence Features of B*27:04 Ligands Do Not Determine Their Differential ERAP1-dependent Expression—We investigated whether peptide residues other than P1 might account for the prevalence of some B*27:04 ligands in a given ERAP1 context. This analysis was carried out only for nonamers and was based on the reported role of residues downstream of P1 in modulating trimming by ERAP1 (29). Each residue at positions P3 to P9 (as P2 was nearly invariant among B*27:04 ligands) was assigned a score related to its

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### Table II

**Relationship between Mw and length among 372 B*27:04 ligands**

| Mw       | <9-mers<sup>a</sup> | 9-mers | 10-mers | >10-mers<sup>c</sup> |
|----------|---------------------|--------|---------|-----------------------|
|          | N       | %      | N       | %        | N       | %       | N     | %      |
| 850–900  | 3       | 100    | 0       | 0        | 0       | 0       | 0     | 0      |
| 900–1000 | 8       | 21.1   | 30      | 79.0     | 0       | 0       | 0     | 0      |
| 1000–1100| 7       | 5.9    | 102     | 94.1     | 8       | 6.8     | 1     | 0.9    |
| 1100–1200| 0       | 0      | 127     | 100      | 20      | 13.5    | 1     | 0.7    |
| 1200–1300| 0       | 0      | 24      | 87.5     | 19      | 41.3    | 3     | 6.5    |
| 1300–1400| 0       | 0      | 1       | 99       | 4       | 58.3    | 4     | 33.3   |
| 1400–1500| 0       | 0      | 0       | 100      | 0       | 0       | 4     | 100    |
| 1500–1600| 0       | 0      | 0       | 0        | 2       | 100     | 16    | 100    |
| 1600–1700| 0       | 0      | 0       | 0        | 0       | 1       | 100   | 100    |
| Total    | 18      | 4.8    | 284     | 76.3     | 54      | 14.5    | 16    | 4.3    |

<sup>a</sup> Percent values are relative to each Mw range, except in the “Total” line, where the values are relative to the total number of peptide sequences.

<sup>b</sup> All these peptides were 8-mers, except one 7-mer.

<sup>c</sup> 11-mers: 10; 12-mers: 4; 13-mers: 1; 15-mers: 1.

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1.7 ± 0.2 for peptides <9-mers and >10-mers, respectively) (Fig. 5C).

Thus, in WE-I, long B*27:04 ligands are more abundant and short ones are less abundant than in JSL and, to a lesser extent, C1R04. The results indicate that ERAP1 polymorphism in WE-I leads to decreased trimming and affects the expression level of a substantial percentage of the B27 peptidome. The idea that the observed differences are due mainly to ERAP1, rather than to unrelated factors, is strongly supported by the similarity between C1R04 and KNE.

**The Internal Sequence Features of B*27:04 Ligands Do Not Determine Their Differential ERAP1-dependent Expression**—We investigated whether peptide residues other than P1 might account for the prevalence of some B*27:04 ligands in a given ERAP1 context. This analysis was carried out only for nonamers and was based on the reported role of residues downstream of P1 in modulating trimming by ERAP1 (29). Each residue at positions P3 to P9 (as P2 was nearly invariant among B*27:04 ligands) was assigned a score related to its
effect on ERAP1-mediated trimming (supplemental Table S1). For each peptide subset, the mean score of each position and the means of the P3–P9 scores were calculated. Although position-dependent differences were observed (supplemental Table S5), the joint contribution of P3–P9 to trimming was virtually identical in all cases, independent of the relative abundance of the peptide subsets compared (Table IV). These results strongly suggest that, globally, the internal peptide positions do not have a significant influence on the differential expression of B*27:04 ligands in distinct ERAP1 contexts.

**Fig. 5.** Length distribution of B*27:04 ligands as a function of their relative abundance in various ERAP1 contexts. A, WE-I/JSL. The upper histogram shows the length distribution of 3222 shared ligands between both cell lines. The percent of peptides with <9, 9, 10, or >10 residues is indicated. The other four histograms compare the length distribution of the peptide sets predominant (IR > 1.0) in WE-I (white bars) or JSL (black bars) and those of the corresponding subsets showing IR > 3.0, IR > 1.5 to 3.0, and IR > 1.0 to 1.5 in each cell line. The ratio between the percentages of peptides of the same length in both cell lines (WE-I/JSL ratio) is indicated over the corresponding bars. B, WE-I/C1R04. The upper histogram shows the length distribution of the shared ligands between both cell lines. The other four histograms compare the length distribution of the peptide sets predominant in WE-I (white bars) or C1R04 (black bars), following the same conventions as in panel A, and showing the WE-I/C1R04 ratios. The data are means of two experiments. C, C1R04/KNE. The upper histogram shows the length distribution of the shared ligands between both cell lines. The other four histograms compare the length distribution of the peptide sets predominant (IR > 1.0) in C1R04 (black bars) or KNE (white bars), following the same conventions as in panel A, and showing the C1R04/KNE ratios. The data are means of three experiments.

**ERAP1 Polymorphism Influences HLA-B27 Stability—**The molecular stability of B*27:04 expressed in various ERAP1 contexts was analyzed by measuring the thermostability of
Influence of N-terminal flanking and P1 residues of B*27:04 ligands on ERAP1-mediated trimming

| Subsetb | Mean score | WE-I/JSL |   |   |   |   |   |
|---------|------------|----------|---|---|---|---|---|
| IR > 3  | P-2        | 34.2     | 38.4 | 72.6 | 29.9 |
|         | P-1        | 14.4     | 37.9 | 52.3 | 12.0 |
|         | P-1 + P-2  | 2.4      | 1.0  | 1.4 | 2.5 |
|         | P1         |          |      |     |     |
| Ratio   |            | 1.5      | 0.9  | 1.1 | 0.8 |

| Subsetb | Mean score | WE-I/C1R04 |   |   |   |   |   |
|---------|------------|------------|---|---|---|---|---|
| IR > 3  | P-2        | 38.5      | 46.3 | 84.7 | 23.4 |
|         | P-1        | 26.5      | 36.1 | 62.6 | 22.6 |
|         | P-1 + P-2  | 1.5       | 1.3  | 1.4 | 1.0 |
|         | P1         |          |      |     |     |
| Ratio   |            | 0.9       | 1.0  | 0.9 | 1.1 |

| Subsetb | Mean score | KNE/C1R04 |   |   |   |   |   |
|---------|------------|-----------|---|---|---|---|---|
| IR > 3  | P-2        | 33.6      | 42.9 | 75.9 | 19.4 |
|         | P-1        | 30.7      | 36.1 | 66.4 | 23.2 |
|         | P-1 + P-2  | 1.1       | 1.2  | 1.1 | 0.8 |
|         | P1         |          |      |     |     |
| Ratio   |            | 2.7       | 46.3 | 73.6 | 20.7 |

| Subsetb | Mean score | C1R04     |   |   |   |   |   |
|---------|------------|-----------|---|---|---|---|---|
| IR > 3  | P-2        | 25.9      | 36.8 | 62.8 | 21.7 |
|         | P-1        | 27.3      | 43.6 | 77.5 | 19.5 |
|         | P-1 + P-2  | 1.1       | 1.3  | 1.2 | 1.0 |
|         | P1         |          |      |     |     |

a The P-2, P-1, and P1 residues of each peptide were assigned a score (supplemental Table S1) related to their susceptibility to ERAP1 trimming in vivo (28). The mean score for each individual position, or for P-1 + P-2, is shown for each peptide subset. The flanking sequences are shown in supplemental Table S4.

b Peptide subsets whose ion peaks show the indicated intensity ratio (IR) relative to the other cell line.

The internal sequence features of B*2704-bound nonamers do not determine their ERAP1-dependent expression

| Peptide subsetb | Mean P3–P9 score | WE-I/JSL |   |   |   |   |   |
|-----------------|------------------|----------|---|---|---|---|---|
| IR > 3          | P-3–P9           | 2.3      | 2.4  | 2.3 | 2.4 |
| IR > 1 to 3     | P-3–P9           | 2.5      | 2.5  | 2.5 | 2.5 |

a Each residue at a given position was assigned a score (supplemental Table S1) related to its effect on ERAP1-mediated trimming (29). The mean added score of positions P3 to P9 for each peptide subset, as well as the corresponding ratios, are given (see supplemental Table S5 for full details).

b Peptide subsets whose ion peaks show the indicated intensity ratio (IR) relative to the other cell line.

The role of natural ERAP1 polymorphism in shaping the HLA-B27 peptidome was addressed in this study. We determined quantitative effects on the expression of B27 ligands, assuming that differential processing among ERAP1 variants may affect many peptides. We did not look for specific ligands whose presence might depend on a particular ERAP1 context, because the absence of a peptide cannot be formally established, and there are no likely candidates for AS-specific epitopes.

Our experimental approach, which was a powerful one for detecting general effects on the peptidome, deserves some comments. The intensity of an ion peak in MALDI-TOF MS is influenced by many factors, including instrument settings, sample handling, the presence of other components, etc. For this reason, although the acquisition parameters and sample preparation were strictly controlled, the IR of a given ion peak from two peptide pools is not a quantitative measurement; it is only an estimation of relative amounts. Yet, when this method is applied to many ion peaks, collective tendencies can be revealed. Thus, our data should be interpreted as general patterns within large peptide sets, and not on an individual peptide basis. MALDI-TOF MS was used in earlier studies to estimate expression differences of HLA-B27 ligands in the absence or presence of tapasin (36) or among HLA-B27 subtypes (32). A second issue concerns the scores used. We based our score system for flanking and P1 residues on an assay that analyzed antigen presentation as a function of ERAP1 processing in the ER (28), rather than in vitro trimming of synthetic substrates, which might not accurately reflect trimming in vivo. To our knowledge, only one systematic study (29) analyzed the effect of internal peptide residues on ERAP1 trimming. It allowed us to examine the sequence of B27 ligands for their influence on ERAP1-dependent peptide expression. That study used nonamer libraries and did not include all residues at each position, which precluded an exhaustive scoring and restricted our analysis to nonamers. Again, although not fully accurate for individual
ligands, this was suitable for globally assessing extensive peptide sets.

Many ERAP1-independent factors, including other peptidases, can influence the relative expression of MHC-I ligands among cell lines. However, most of these factors should not imply cell-specific effects on peptide size, because no other amino peptidases, except perhaps IRAP (18), are regulated by substrate length, and this enzyme is most likely not involved in the shaping of endogenous MHC-I peptidomes (17). Thus, cells expressing similar ERAP1 molecules might be expected to generate B*27:04 peptidomes with a similar size distribution. That this was the case between C1R04 and KNE strongly suggests that the significant size differences observed in the context of more distinct variants were mostly due to ERAP1 polymorphism. Indeed, the ERAP1 activity ranking deduced from the effects on the size and length of B27 ligands, JSL/C1R04/KNE, correlated with the number of amino acid differences among the ERAP1 molecules in these cell lines.

Differences in ERAP1 expression levels among cell lines are very unlikely to account for the observed differences among the HLA-B27 peptidomes, given that WE-I and C1R04, which expressed very similar ERAP1 amounts, showed significantly larger size differences in their B27-bound peptidomes than C1R04 and KNE, with larger ERAP1 protein expression differences. Furthermore, JSL showed the lowest expression of ERAP1 mRNA among the four cell lines, but it had the most efficient ERAP1 trimming on the B27 peptidome.

What is the mechanism by which ERAP1 polymorphism modulates the expression level of B*27:04 ligands? What peptides are more likely to be affected? ERAP1 can remove almost any N-terminal residue of peptides longer than a minimum length, albeit with different efficiencies (28). Therefore, ERAP1 variants with different enzymatic activity might show differences in the generation of MHC-I ligands as a function of their N-terminal flanking extensions. A previous study (37) showed that although residue P-3 and those more distant can probably be removed by a variety of peptidases in vivo, P-2 and P-1 are predominantly removed in the ER. Moreover, activity differences among ERAP1 variants will affect epitope destruction, because the P1 residue will be more efficiently cleaved by the most active variant. We have directly observed this effect with synthetic peptides in vitro (our observations remain unpublished). The higher susceptibility to trimming of flanking and P1 residues in the peptide subset most predominant in WE-I, relative to its equivalent in JSL, implies that these positions would be trimmed more efficiently by the more active ERAP1 variant in JSL, so that the corresponding ligands would be not only generated more efficiently but also destroyed to a greater extent in the latter cell line. The abundance of these peptides in WE-I would be explained by the presence in this cell line of a less active ERAP1 capable of removing susceptible flanking residues without extensively destroying the epitope. Many peptides predominant in JSL would be generated less efficiently in WE-I because of the lower susceptibility of their flanking residues to trimming.

The same mechanism would explain the observed differences between WE-I and C1R04, but here the similar susceptibility of P1 residues to trimming in the predominant peptide subsets from both cell lines means that the more active ERAP1 in C1R04 would still destroy B27 ligands more extensively than in WE-I, but less than in JSL. This might explain why the size differences between the B27 peptidomes from WE-I/C1R04 are smaller than in WE-I/JSL.

In conclusion, natural ERAP1 polymorphism has a significant influence on the length and abundance of many HLA-B27 ligands. The less active variant generated higher numbers of long peptides and less short ones, and it also increased the

**Fig. 6.** Thermostability of HLA-B*27:04 in various ERAP1 contexts. The indicated cell lines were labeled for 15 min and chased at 0, 2, and 4 h. Equal aliquots of the lysates were kept at 4 °C or heated at the indicated temperatures for 1 h prior to immunoprecipitation with ME1 (a mAb that recognizes undissociated HLA-B27/peptide complexes but not unfolded HC), separated via SDS-PAGE, and analyzed using fluorography. The percentage of ME1-reactive HLA peptide complexes recovered at 0 h (○), 2 h (□), or 4 h (△) chase after heating was plotted as the intensity of the class I HC at any given temperature (HCt) relative to that at 4 °C (HC4°C). The data are means ± S.D. of four (C1R04), five (KNE), and eight (WE-I) experiments. Only one experiment could be performed with JSL. The data concerning C1R04, except for one additional experiment included here, were reported previously (35) and are shown here only for comparison.
expression of peptides of any length with susceptible flanking and/or P1 residues. Thus, the variant-dependent alteration in the balance between epitope generation and destruction, as a function of these residues, is a basic mechanism determining ERAP1-dependent differences among HLA-B27 peptidomes. Although the internal sequence features of B*27:04 ligands did not globally affect relative peptide expression in distinct ERAP1 contexts, our results do not exclude an influence of individual sequences on ERAP1 trimming, as reported in vitro (29). The general character of the effects observed among the four cell lines in our study should be established by extending these analyses to additional cell lines showing similar ERAP1 variants, as well as distinct ones.

Several studies have analyzed the effect of natural polymorphisms on the enzymatic activity of ERAP1. The K528R and R725Q mutations, both of which are protective with regard to AS, decreased ERAP1 activity, whereas R127P, I276M, D575N, and Q730E had a smaller or no effect (10, 20, 38). These studies were performed with single mutants in vitro, and the mutual influence of co-existing mutations or their effects in vivo were not addressed. In a recent study (39), the kinetics of ERAP1 activity was consistent with a substrate-inhibition model that was both substrate and allele specific. The K528R and Q730E changes at these positions.

The effects of ERAP1 polymorphism described here might influence HLA-B27 biology in various aspects relevant to AS pathogenesis. In the discussion that follows, we comment on the incidence of this study on the current hypotheses concerning the pathogenetic role of HLA-B27. First, on the basis of our results, ERAP1 polymorphism can affect the immunological properties of the HLA-B27 heterodimer at various levels. The differential effects on trimming observed among ERAP1 variants might result in the production (or not) of some B27 ligands. As protection from AS has been linked to ERAP1 mutations leading to decreased enzymatic activity (10), one might speculate whether these studies whether association with AS is determined by a single position or by co-occurring polymorphisms in the susceptible haplotypes.

Genetic analyses are consistent with a two-mutation model in which the association of ERAP1 with AS is determined by a primary effect of rs30187 (K528R) and a secondary effect of rs17482078 (R725Q) and/or rs10050860 (Q730E), two polymorphisms in very tight linkage disequilibrium. In combination, the protective alleles at these loci (R528, Q725, E730) confer particularly strong protection from AS (10). Among the cell lines in our study, only WE-I carried these three protective polymorphisms; C1R04 and KNE showed only two (R528, E730), and JSL carried all three susceptibility allotypes (K528, R725, Q730). Thus, without ruling out an additional contribution of other positions, the observed effects of ERAP1 polymorphism on HLA-B27 in our cell lines are fully consistent.
active variants might be peptides with flanking and/or P1 residues not highly susceptible to ERAP1 trimming. Moreover, altering the expression level of many B27 ligands is likely to influence T-cell repertoire selection, which is determined by the avidity of TCR/peptide/MHC interactions and, therefore, by individual epitope amounts at the cell surface. Thus, the autoimmune potential of HLA-B27 might be modulated by ERAP1 polymorphism. Finally, HLA-B27 immunogenicity would also be modulated by ERAP1 through its quantitative effect on the peptidome, because T-cell triggering thresholds are dependent on the number of peptide/MHC complexes within individual TCR clusters during T-cell activation (44).

Second, although the influence of ERAP-1 on HLA-B27 folding was not examined, the decreased molecular stability of HLA-B27 observed in WE-I indicates that certain ERAP1 variants containing a number of AS-protective polymorphisms generate suboptimal B27 peptidomes. We have shown previously that high thermostability is a feature of three AS-associated subtypes (B*27:02, B*27:04, and B*27:05) expressed on C1R cells that is not shared by the non-AS-associated B*27:06 and B*27:09 subtypes or the AS-associated B*27:07 and B*1403 (30, 35). The thermostability of B*27:04 in WE-I was closer to that of the latter subtypes, indicating that ERAP1 polymorphism protective against AS can approach the molecular stability phenotype of an AS-associated B27 subtype to that shown by non-AS-associated ones. Decreased thermostability by itself does not correlate with AS susceptibility. However, the molecular mechanism leading to decreased stability in B*27:06, B*27:07, B*27:09, and B*1403 is probably different than that operating in B*27:04 from WE-I. Indeed, the four former subtypes have a hydrophobic F pocket and bind almost exclusively peptides with nonpolar C-terminal residues (34, 45, 46). As shown for HLA-B*44 subtypes (47, 48), increased hydrophobicity of the F pocket leads to intrinsic thermodynamic instability of the empty molecule that favors quick loading of hydrophobic peptides to hide nonpolar regions from exposure to water. Presumably, this mechanism might largely bypass tapasin-mediated peptide editing, leading to fast folding, albeit with a suboptimal peptide cargo. Indeed, B*27:06, B*27:07, B*27:09, and B*1403 did not misfold significantly in the ER (30, 35). The lower stability of B*27:04 in WE-I is not obviously due to altered hydrophobicity of its peptide binding site, because the molecule itself is not altered; rather, presumably, it is due to the inability of the ERAP1 variant to generate an optimal peptide repertoire. If so, thermodynamic factors favoring quick peptide binding would not play a differential role in WE-I relative to other B*27:04-positive cell lines, and tapasin might not be bypassed. Rather, if an optimal peptide pool is not available because of ERAP1 impairment, this might result in slower loading kinetics and perhaps increased B*27:04 misfolding in the context of an ERAP1 variant with multiple protective polymorphisms, in contrast with the HLA-B27 misfolding hypothesis for AS pathogenesis. Although this prediction remains to be confirmed for B*27:04 in WE-I, it is consistent with the association of B*27:07 and B*1403 with AS, despite their presumably low misfolding (30, 35).

The expression of HLA-B27 HC homodimers at the cell surface has been proposed to have pathogenetic significance because of their immunomodulatory potential through their recognition by leukocyte receptors (49). These homodimers are formed upon endosomal recycling of HLA-B27 (50) and presumably arise from dissociation of the canonic heterodimer. If, as shown in this study, ERAP1 polymorphism associated with protection from AS can decrease HLA-B27 stability, one might expect that ERAP1 changes associated with AS susceptibility would lead to decreased expression of B27 HC homodimers. In agreement with this prediction, a recent report (51) showed decreased reactivity with HC10, an antibody that recognizes unfolded MHC-I HC, including homodimers, at the surface of monocytes from B*27:05-positive individuals homozygous for Q730 in ERAP1. This change is associated with AS susceptibility and efficient ERAP1 trimming in vivo (39). Again, the effects of AS-associated ERAP1 polymorphism seem to affect HLA-B27 in a way opposite that expected from the surface homodimer pathogenic hypothesis.

Given that the effects of ERAP1 on the B27 peptidome are essentially dependent on the flanking and P1 residues of the HLA-B27 ligands, these effects should presumably be similar for most of the main HLA-B27 subtypes, because they have overlapping peptide repertoires and similar residue usage at P1 (45). An exception is B*27:03, whose special preference for basic P1 residues (52–54), which are relatively resistant to ERAP1, might lead to decreased epitope destruction (relative to that in other subtypes) by ERAP1. More generally, different P1 residue usage by diverse HLA-I molecules might significantly affect the way in which natural ERAP1 polymorphism shapes their peptidomes.

A different issue is the effect of ERAP1 polymorphism on the stability of other HLA-B27 subtypes. Our results suggest that AS-protective polymorphism might decrease the stability of those subtypes that bind highly optimized peptidomes, such as the AS-associated B*27:02 and B*27:05. However, these effects might not be comparable for B*27:06, B*27:07, B*27:09, and B*1403, whose binding of suboptimal peptidomes is thermodynamically determined by the hydrophobicity of their peptide-binding grooves. A smaller influence of ERAP1 polymorphism on the molecular stability of these subtypes might be expected.

In conclusion, our results reveal large effects of natural ERAP1 polymorphism on the HLA-B27 peptidome in vivo, as well as their molecular basis. These effects, through their influence on immunological and other features of HLA-B27, define the nature of the functional interaction between both molecules, underline the fundamental role of peptides in the pathogenesis of HLA-B27-associated disease, and suggest...
that this role is mainly to alter the immunological specificity of the HLA-B27 heterodimer.  

Acknowledgements—We thank the staff of the Proteomics facility at the Centro Nacional de Biotecnología, Madrid, and Ricardo Ramos (Parque Científico de Madrid) for help in MS and DNA analyses, respectively.

This work was supported by grants SAF2008/00461 and SAF2011/25681 from the Plan Nacional de I+D+i, RD08/0075 (RIER) from the Instituto de Salud Carlos III, and an institutional grant from the Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa.

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ψ This article contains supplemental material.

This article was published in the 11.11 issue of Molecular & Cellular Proteomics.
ERAP1 and the HLA-B27 Peptidome

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