Combined effects of ankylosing spondylitis-associated ERAP1 polymorphisms outside the catalytic and peptide-binding sites on the processing of natural HLA-B27 ligands

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*The contribution of AME and PGM to this work was equal

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*Running title: Combined effects of disease-associated ERAP1 polymorphism

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Background: The endoplasmic reticulum aminopeptidase (ERAP)1 is associated with HLA-B27+ ankylosing spondylitis.

Results: ERAP1 polymorphism at residues 528/575 affects the processing of HLA-B27 ligands in variant-, peptide- and mutually-dependent ways.

Conclusion: ERAP1 shapes HLA-B27 peptidomes through combined effects of co-occurring polymorphisms.

Significance: ERAP1-induced, peptide-mediated alterations of the immunological/pathogenetic features of HLA-B27 explain the epistasis of both molecules in ankylosing spondylitis.

ABSTRACT

ERAP1 polymorphism involving residues 528 and 575/725 is associated with ankylosing spondylitis among HLA-B27-positive individuals. We used four recombinant variants to address the combined effects of the K528R and D575N polymorphism on the processing of HLA-B27 ligands. The hydrolysis of a fluorogenic substrate, R528/D575<K528/D575<R528/N575<K528/ N575, indicated that the relative activity of variants carrying R528 or K528 depends on residue 575. D575 conferred lower activity than N575, but the difference depended on residue 528. The same hierarchy was observed with synthetic precursors of HLA-B27 ligands, but the effects were peptide-dependent. Sometimes epitope yields were variant-specific at all times. For other peptides, concomitant generation and destruction led to similar epitope amounts with all the variants at long, but not at short, digestion times. The generation/destruction balance of two related HLA-B27 ligands was analyzed in vitro and in live cells. Their relative yields at long digestion times were comparable to those from HLA-B27-positive cells, suggesting that ERAP1 was a major determinant of the abundance of these peptides in vivo. The hydrolysis of fluorogenic and peptide substrates by an HLA-B27 ligand or a shorter peptide, respectively, was increasingly inhibited as a function of ERAP1 activity, indicating that residues 528 and 575 affect substrate inhibition of ERAP1 trimming. The significant and complex effects of co-occurring ERAP1 polymorphisms on multiple HLA-B27 ligands, and their potential to alter the immunological and pathogenetic features of HLA-B27 as a function of the ERAP1 context, explain the epistatic association of both molecules in ankylosing spondylitis.

ERAP1 is a multifunctional aminopeptidase of the endoplasmic reticulum (ER) involved in the final processing steps of Major histocompatibility Complex class I (MHC-I) ligands. Its role in this pathway is to trim peptides to the optimal size for MHC-I binding, which is 9-10 amino acid residues (1-3). Various non-synonymous polymorphisms, involving amino acid
changes in the protein (4,5), are associated with ankylosing spondylitis (AS), a chronic form of arthritis typically affecting the axial skeleton and very strongly associated with HLA-B27 (6,7). Due to strong linkage disequilibrium (LD) between polymorphisms within the same gene it is often difficult to discern the contribution of each mutation to AS susceptibility or protection. Yet, it has been determined that this association fits a two mutation model with contributions of both the single nucleotide polymorphisms (SNPs) rs301087 (coding for K528R) and rs10050860/rs17482078 (coding for D575N/R725Q), the two latter changes being indistinguishable due to tight LD (8). The fact that the association of ERAP1 with AS concerns only the HLA-B27-positive disease (8,9) strongly suggests that it is based on the functional interaction between ERAP1 and HLA-B27. In a recent study (10) we showed that the AS-associated natural polymorphism of ERAP1 variants altered the expression level of many HLA-B27-bound peptides in live cells. On the basis of that study we proposed that the mechanism of functional interaction between these two proteins, and presumably the basis for their joint association with AS, is the alteration in the balance between epitope generation and destruction induced by ERAP1 polymorphism on the HLA-B27 peptidome.

Several in vitro studies using recombinant ERAP1 mutants and synthetic peptides have shown that the AS-protective changes R528, Q725 and E730 result in decreased enzymatic activity, whereas no effect on trimming was reported for R127P or D575N using single-residue mutants (8,11,12). In these studies few peptides were tested. Since ERAP1 activity is influenced by the N-terminal residue of the substrate (13) and its sequence downstream the N-terminus (14), an incomplete and potentially misleading picture of the effect of a mutation might be obtained. In addition, the use of single mutants precluded analyzing possible combined effects among co-occurring mutations. This is particularly relevant, since natural ERAP1 variants are complex allotypes that differ among each other by multiple amino acid changes (15-17), whose individual contribution to disease is not always distinguished by genetic analyses and whose concurrent influence on ERAP1 activity needs to be directly tested. Indeed, significant specificity differences, including some mediated by residue 575, were reported among natural ERAP1 variants with diverse combinations of amino acid changes, reflecting complex interactive effects in ERAP1 haplotypes on enzymatic activity (18).

In the present study four recombinant ERAP1 variants and a panel of synthetic peptides were used to analyze the context-dependent contribution of the AS-associated changes K528R and D575N to the processing of natural HLA-B27 ligands. We focused on these two residues for the following reasons: 1) as mentioned above, both K528R and D575N and/or R725Q are critical in the two-mutation model that explains the genetics of ERAP1 association with AS (8), 2) contrary to residue 725, which is known to affect ERAP1 activity (8), there are conflicting reports concerning D575N; whereas two studies (8,11) found no effect of this mutation, a recent one (18) found a positive effect, and 3) both residues 528 and 575 are located outside the catalytic and peptide-binding sites of the enzyme, and are thought to influence the domain rearrangements associated with the acquisition of an active ERAP1 conformation (19,20). These three features are not fulfilled for other AS-associated polymorphisms. Two issues were specifically addressed: 1) the influence of each mutation on the generation and destruction of natural HLA-B27 ligands, and 2) how the effect of a given residue in one position on ERAP1 activity and in the generation/destruction balance of HLA-B27 ligands was influenced by the residue at the other position.

EXPERIMENTAL PROCEDURES

ERAP1 variants--The starting ERAP1 construct (a kind gift of Dr. S. C. Chang, National Taiwan University) was a pFastBac/myc-His-ERAP1 plasmid containing a full length cDNA coding for an ERAP1 variant that, upon re-sequencing in our laboratory, showed four non-synonymous substitutions (coding for D346, R514, R528 and E730) relative to the GenBank reference sequence NP-057526. Three additional ERAP1 variants (Table I) were generated from the initial construct, hereby termed wildtype, by PCR-mediated site-directed
mutagenesis of codons 528 and 575. The primers used were: for the R528K mutation: 5’-CACCTGACACTGCAGAAGGGTTTTCCCTAAT AACC-3’ (sense) and 5’-GGTTATTAGGGGAAAACCCTCTGCAGTGCA ACTG-3’ (antisense); for the D575N mutation: 5’-GACATTCACTCACGCAAATCCCAACATGGTCCA TCAGATTITGC-3’ (sense) and 5’-GCAAAAATCAGTGGACATGTTGGATTTGCTGG TGATGAATGTC-3’ (antisense).

To our knowledge the variants in this study are not identical to any known natural allotypes. However, all the individual amino acid changes encoded in the four constructs are naturally occurring polymorphisms and it is known from reported natural variants (10,18) that individual polymorphisms occur in very diverse combinations. Thus, it is likely that the variants analyzed here may correspond to as yet undetected natural ones.

Generation of recombinant baculovirus--The correct products were confirmed by sequencing and subsequently used to transform competent DH10Bac E.coli. Recombinant bacmids were isolated by standard DNA preparation methods and used to transfect Hi-5 adherent insect cells with Cellfectin II (both from Invitrogene, USA) to produce the recombinant baculoviruses. These were harvested from the cell supernatant after 72 h and its titer determined by an immunofluorescence plaque assay with a mouse anti-His Ab (Quiagen, Hilden, Germany) and an Alexa 488 donkey anti-mouse Ab (Invitrogene), at 1:400 and 1:500 dilutions, respectively. Larger amounts of viruses were produced by infecting Hi-5 adherent cell cultures at high multiplicity of infection and collecting the supernatant after 72 h.

Protein expression and purification--Recombinant ERAP1 proteins were produced in non-adherent Hi-5 insect cells grown in Express Five serum-free medium (Gibco, Grand Island, NY). After infection with baculovirus carrying the ERAP1 gene construct, the culture medium containing the secreted enzyme was harvested by centrifugation (3000xg, 30 min, 4°C). The supernatant was concentrated in a Stirred Ultrafiltration Cell (Amicon, Millipore, USA), adjusted to 50 mM phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0, and loaded onto a Poly-prep Chromatography Column (Bio-Rad, Hercules, CA) pre-loaded with Ni-NTA-Agarose (Qiagen). The column was washed with the same buffer, containing 20 mM imidazole, and the protein was eluted with a 40 to 150 mM imidazole gradient. Protein elution was checked by SDS-PAGE. Protein-containing fractions were dialyzed in Vivaspin 500 (Sartorius Stedim Biotech, Goettingen, Germany) against 50 mM Tris/HCl buffer, 1mM DTT, pH 7.4, aliquoted and stored at -70°C.

Fluorogenic substrate hydrolysis assay--Three µg of Leu-7-amido-4-methyl-coumarine (L-AMC) (Bachem Distribution, Wei an Rhein, Germany) in 50 μl of 1M Tris/HCl buffer, pH 8.0 was mixed with 100 ng of ERAP1 in an equal volume of 50mM Tris/HCl, 1mM DTT, pH 7.4 (E:S ratio, 1:30) and incubated at 37°C up to 1h time. The substrate hydrolysis was assessed by measuring its fluorescence, at 14 sec intervals, at 380 nm and 460 nm excitation and emission wavelengths, respectively, in a Fluostar Optima Multiwave Plate Reader (BMG Labtech, Ortenberg, Germany).

Synthetic peptides--A total of 10 peptides, including 8 precursors of prominent natural HLA-B27 ligands and 2 fully processed ones (Table II) were obtained using standard N-(9-fluorenyl)-methoxycarbonyl chemistry and purified by HPLC (purity >80%). The correct m.w. and sequence of the synthetic peptides were confirmed by MALDI-TOF mass spectrometry (MS) using a 4800 Proteomics Analyzer (Applied Biosystems).

Peptide trimming assays--One µg of each peptide was incubated with 100 ng of ERAP1 (E:S ratio 1:10) in 50μl of 50mM Tris/HCl buffer, 1mM DTT, pH 7.4 at 37°C at various times from for 5 min to 4h. After incubation, the reaction was stopped with 5µl of 5% trifluoroacetic acid. The peptides in the reaction mixtures were purified with OMIX C18 pipette tips (Varian Inc. Palo Alto, CA) following the instructions of the manufacturer. The samples were dried down in speed-vac, dissolved in 1μl of 30% acetonitrile, 15%, isopropanol and 0,1% trifluoroacetic acid, and sonicated for 3 minutes. The digestion mixtures were analyzed by MALDI-TOF MS in positive ion reflector mode at 25kV in the mass-to-charge (m/z) range of 400-2200 as previously
described (21). Peptide yields were estimated on the basis of the relative intensity of the respective ion peaks.

MS was used instead the more conventional HPLC due to the significantly higher sensitivity of the former method and to the difficulties of separating similar and co-eluting peptide species in the relatively complex digestion mixtures generated from the long precursors used in our study. Yet, to confirm the reliability of MS-based estimations, we carried out pilot experiments to assess the correspondence between both detection methods. In those experiments the precursor peptide 7 (LREI.RRYQKSTEL) was digested with R528/D575 or K528/N575 and analyzed at every time point by MS and HPLC.

Identification of HLA-B27 ligands in live cells--This was performed exactly as previously described (10). Briefly, HLA-B27-bound peptides were isolated from C1R-B*27:04 or -B*27:05 transfectant cells and from the B*27:04 lymphoblastoid cell line Wewak I by immunopurification of HLA-B27 and acid extraction, and fractionated by HPLC. Each chromatographic fraction was analyzed by MALDI-TOF MS, and individual peptides were identified by MS/MS sequencing.

RESULTS

Context-dependent effects of AS-associated ERAP1 polymorphism on L-AMC hydrolysis--The relative activity of four ERAP1 variants differing at residue 528 (K/R), 575 (D/N) or both (Table I) was assessed by measuring the hydrolysis of the fluorogenic L-AMC substrate as a function of time (Fig. 1). Substantial differences were observed with the following activity ranking: K528/N575 > R528/N575 > K528/D575 > R528/D575. These results indicate that: 1) K528 confers higher activity than R528 if residue 575 is the same, 2) N575 confers higher activity than D575 regardless of residue 528, 3) the relative activity of variants differing by the K528R change depends on residue 575: K528/N575 > R528/N575 and K528/D575 > R528/D575, but R528/N575 > K528/D575, and 4) this is due to the fact that the D575N mutation increased ERAP1 activity more than R528K in this system.

ERAP1 polymorphism at residues 528 and 575 influences the generation and destruction of HLA-B27 ligands in a variant and peptide-dependent way--The effect of AS-associated polymorphism at each or both positions on the generation and destruction of natural HLA-B27 ligands was analyzed with 10 peptide substrates (Table II). Peptides 1 to 6 were 16-mer precursors of natural ligands, including 4 nonamers and 2 decamers. In addition, two natural ligands, a 9-mer and a C-terminally extended 12-mer variant (peptides 9 and 10), as well as their N-terminally extended precursors with 4 flanking residues (peptides 7 and 8) were used. This panel was chosen to favor diversity of N-terminal and C-terminal residues, as well as internal sequences, in order to assess peptide-dependent differences in ERAP1 trimming. The flanking and P1 residues were assigned a score (1 to 4) based on the susceptibility of each residue to ERAP1 (Table II). The peptides were digested at various times up to 4h, since longer incubation times usually yielded little further digestion.

In the following analyses (Fig. 2) we computed the combined digestion of all the species longer than the natural ligand excluding the original substrate, designated as epitope precursors, the yield of the ligand and the yield of shorter species, resulting from destructive cleavages, as a function time. The cumulative digestion of epitope precursors was chosen because the digestion rate of the enzyme is strongly dependent on the N-terminal residue of the substrate, so that the generation of the natural epitope will depend on the combined digestion rate of all the precursor species. The yield of the ligand at each time point is the epitope amount generated minus that destroyed at that time point.

To validate the reliability of the MS-based assessment of yields of digestion products we performed two comparative experiments in which one the substrates in Fig. 2 (peptide 7) was digested with R528/D575 or K528/N575 and analyzed by MALDI-TOF MS and HPLC (Fig. 3). The results show that digestion rates and relative yields are similar with both techniques. The small differences observed are due to the higher sensitivity of MS that allows detection.
of peptide species present in very low amounts that go undetected by HPLC.

**Digestion of epitope precursors**--The four ERAP1 variants showed distinct patterns of digestion that were both peptide and variant dependent (Fig. 2A). Distinct peptides were digested with very different rates and efficiencies by any given variant. The relative efficiency with which the 4 variants digested a given substrate also differed widely among peptides. Yet, the average values of the maximal digestion of epitope precursors from peptides 1 to 8 increased in the following order: R528/D575 (36.1%) < K528/D575 (41.7%) < R528/N575 (56.3%) < K528/N575 (84.3%). Thus, the relative activity of the 4 ERAP1 variants towards peptidic substrates is the same as established with L-AMC. Peptide-dependent differences in the digestion of precursors correlated with the susceptibility of the flanking sequences to ERAP1 trimming (Table II). These differences were much larger with the less active variants R528/D575 and K528/D575 (6.8-79.8% and 3.6-72.8%, respectively) than with the most active one, K528/N575 (74.5-99.9%). Thus, resistance of the flanking sequences to ERAP1 has a larger influence on the digestion of epitope precursors by less active variants. This is clear, for instance, when comparing peptides 1 and 2 with peptide 6 (Fig. 2A and Table II).

**Destructive cleavages**--The rate and efficiency of epitope destruction was estimated by measuring the combined yield of species shorter than the natural epitope. In general, the patterns of epitope destruction (Fig. 2C) paralleled those of precursor digestions (Fig. 2A), showing similar variant and peptide-dependent differences. In addition, the mean maximal yield of species shorter than the natural HLA-B27 ligands for peptides 1 to 8 paralleled that of the digestion of precursors for each ERAP1 variant, revealing the same activity ranking (Table III): R528/D575 (22.5%) < K528/D575 (26.8%) < R528/N575 (42.1%) < K528/N575 (75.9%). These results indicate that, in general, there is a concomitant and similar effect on both epitope generation and destruction resulting from the alterations in enzymatic activity induced by ERAP1 polymorphism.

**Generation of HLA-B27 ligands**--All the natural ligands were generated with the 4 ERAP1 variants, albeit with widely different efficiencies (Table IV). Due to the concomitant increase of cleavages leading to generation and to destruction of the epitopes as a function of ERAP1 activity, the mean maximal yield of the 8 ligands was similar for R528/D575 (14.0%) and K528/D575 (12.5%) and only slightly higher with the most active ones, R528/N575 (17.9%) and K528/N575 (19.2%). The average time at which the maximal yield of epitopes was obtained was longest with the less active variant R528/D575 (101.9 min) and shortest with the most active one K528/N575 (35.6 min). A similar activity ranking was reflected in the mean earliest time at which the natural ligands were detected in the digestion mixtures (Table IV).

Significant peptide-to-peptide and variant-dependent differences were observed in the maximal amount of epitope and, more importantly, in the time at which this was obtained (Fig. 2B). Four situations were distinguished. For peptides 1, 3, 5 and 7 the highest epitope yields were obtained with the most active enzyme, K528/N575, at shorter times, whereas the tendency was reversed at longer times due to more efficient destruction of the epitope by this enzyme. A second pattern was observed with peptides 4 and 8. For these two substrates the epitope yields were similar with R528/D575, K528/D575 and R528/N575, and consistently higher with K528/N575 at all times. A third pattern concerned substrate 2. Again the highest epitope yield was obtained at the shortest time with K528/N575, although the rate of production was slower than for all other peptides. Significant epitope amounts were also produced with R528/N575, but at a slower rate. The less active enzymes produced the epitope only at the longest digestion times. Finally, substrate 6 generated significant amounts of epitope only with the less active enzyme, R528/D575. With the 3 other variants the epitope was detected in very low amounts and only at short times. This pattern was due to the very efficient digestion of substrate 6 by all four variants. However, whereas the digestion of the epitope precursors was slightly higher with R528/D575 (Fig. 2A), the destruction of the natural ligand by this variant was less efficient (Fig. 2C). Thus, the generation/destruction balance favored...
epitope production by the less active variant in this case. Significantly, peptide 6 had, among those in this study, the flanking and P1 residues most susceptible to ERAP1 trimming (Table II). The earliest times at which the natural ligands were detected and their yields at these times, also showed significant peptide-to-peptide and variant-dependent differences and reflected the same activity ranking among the four variants (Table IV).

In summary, these results indicate that: 1) the four ERAP1 variants differ in their enzymatic activity towards peptide substrates with the same ranking as determined with L-AMC, 2) globally, both the cleavages leading to generation and to destruction of the natural ligands increase with the enzymatic activity, 3) as a result, the mean maximal yield of the HLA-B27 ligands was rather similar for all four variants, 4) significant differences were observed in the processing of individual substrates, both in the way (rate and yield) in which a same variant processed different substrates and in the way in which different variants processed a same substrate, 5) with only one exception epitope production was significantly higher at shorter times with the most active enzyme whereas at longer times differential epitope destruction by the different variants tended to decrease or even reverse relative epitope yields, and 6) in one out of 8 substrates analyzed the generation/destruction balance favored the preferential production of the natural ligand by the less active ERAP1 variant.

Generation of peptide length variants in vitro--Related natural ligands differing in length by C-terminal extensions are frequent in the HLA-B27 and other MHC-I peptidomes. An example is RRYQKSTEL, a prominent ligand of B*27:05 and other HLA-B27 subtypes (22), and its C-terminally extended variant RRYQKSTELLIR, found in B*27:04 (10) and B*27:05 (Figure 2C), as expected from the larger length of RRYQKSTELLIR. Thus, the yield of the shorter epitope RRYQKSTEL was significantly higher than that of RRYQKSTELLIR with all four enzymes (Figure 2B). With the most active variant the maximal yield of epitope 7 was obtained at short times and decreased later due to further digestion. With the three other variants the longer epitope was generated at faster rate, so that at the shortest times (up to 15 min) the yield of the longer ligand (about 3 to 7 %, depending on time and enzyme variant) was comparable or even higher than the yield of the shorter one (about 1 to 5%).

The direct digestion of the natural ligands, peptides 9 and 10, showed dramatic differences (Fig. 5). RRYQKSTEL showed a significant resistance to ERAP1. At the longest digestion time only the most active variant fully digested the nonamer, whereas with the three other enzymes, about 35 to 55% percent of the peptide remained undigested. At shorter times, such as 60 min, as much as 40% to 88% of the ligand remained undigested with any given variant. In contrast, RRYQKSTELLIR was completely degraded by all 4 variants at 180 min of digestion. It was already fully digested after 15 min by K528/D575 and R528/N575 and about 85% by K528/N575. Only the less active variant, R528/D575 showed a significantly lower degradation rate with this peptide, which was, nevertheless much faster than for RRYQKSTEL.

Generation of peptide length variants in live cells--We asked to what extent the more efficient generation of RRYQKSTEL, relative to RRYQKSTELLIR, observed in vitro might hold in live cells, where other variables can influence the endogenous processing of these peptides. Thus, we examined their recovery in the HLA-B*27:04 and B*27:05 peptidomes isolated from the lymphoid cell lines Wewak I (B*27:04+), C1R-B*27:04 and C1R-B*27:05. The endogenous ERAP1 variants of these cell lines (10) are not identical to any of those used in vitro, but ERAP1 in C1R has R528+D575 and in Wewak I has R528+N575 (Table I). The HLA-B27 peptidome isolated from each cell line was fractionated by HPLC
and each fraction was analyzed by MALDI-TOF MS. Each of the peptides was identified by MS/MS sequencing of the corresponding ion peak in the MALDI-TOF spectrum (Fig. 4). The added intensity of the corresponding ion peak in the consecutive HPLC fractions in which the eluted peptide was detected was taken as an estimation of its abundance. Due to the non-quantitative nature of MALDI-TOF MS, the measurements were carried out in 8 independent preparations from C1R-B*27:04, 4 from Wewak I and 3 from C1R-B*27:05 and the average intensity of the ion peak corresponding to each of the peptides in all the experiments was calculated for each cell line. The ratio between the average ion peak intensity obtained for the 9-mer and the 12-mer was taken as an estimation of the relative recovery of both peptides in each cell line and this was compared with the yield of the corresponding peptides upon in vitro digestion of their precursors by R528/D575 and R528/N575. The 9-mer was recovered with higher yield than the 12-mer in ratios of about 4- to 8-fold from the 3 cell lines. These ratios were very similar to those obtained in vitro at 60 min or larger digestion times (Table V). These results suggest that, in spite of the many variables that can influence the amount of MHC-I ligands in vivo, the generation of RRYQKSTEL and RRYQKSTELLIR by ERAP1 may be a major determinant of their abundance in live cells.

**ERAP1 polymorphism influences the inhibition of peptide processing by short peptides**--Since short peptides inhibit the processing of longer substrates (19), we examined whether ERAP1 polymorphism influenced the extent of this inhibition. Peptide 6, a 16-mer that was efficiently processed by all ERAP1 variants (Fig. 2), was tested for the inhibition of its processing in the presence of the RYQKSTEL octamer. The recovery of the precursor substrate after 4h of digestion by the two ERAP1 variants with the largest activity differences, R528/D575 and K528/N575, was analyzed as a function of the octamer concentration (Fig. 7). At high inhibitor concentration (weight ratio 1:1 or higher) the inhibition was similar (19% and 16%) for both enzymes. However, whereas the inhibition fell almost to background levels (6%) at 10:1 substrate/inhibitor ratio for R528/D575, a 1000:1 ratio was required to reach this level with K528/N575. Thus, as with L-AMC, the octamer inhibited peptide trimming by the most active variant more efficiently than for the less active one.

**DISCUSSION**

To assess the contribution of this study to our understanding of the functional ERAP1/B27 interaction in AS three issues must be considered: 1) the influence of ERAP1 polymorphism on the mechanism of peptide trimming, 2) the complexity of natural ERAP1 variants, which usually differ among each other by multiple amino acid residues, and 3) the correspondence of in vitro assays with the situation in live cells.

X-ray diffraction studies (19,20) have virtually confirmed the molecular ruler mechanism of ERAP1 trimming (23), which is dependent on the affinity and length of the
substrate and requires a peptide binding site that is topologically distinct from the catalytic site. A conformational transition, involving global domain movements, is required for optimal enzymatic activity. Thus, ERAP1 polymorphism can influence peptide trimming by affecting: 1) the catalytic site or its immediate environment (i.e: residue 349), 2) the peptide binding site and substrate/enzyme interactions (i.e: residue 730), and 3) the domain rearrangement. Residue 528 is located at the interdomain II-III region, which is the hinge for the domain rotation taking place during the conformational transition. This probably explains the functional effects of K528R. In contrast, residue 575 is located in a loop of 8 residues connecting two β-strands in the middle of domain III, away from interdomain junctions or other functional sites. There are no obvious effects of the D575N mutation on ERAP1 activity that one can infer from just considering its topology. They are not due to changes in the glycosylation pattern, because N575 is not in a consensus glycosylation sequence. Presumably, they are also not due to just an influence on the peptide binding site, since the mutation also affected the hydrolysis of L-AMC, which could explain the observed influence of residue 575 on the hydrolysis of the fluorogenic substrate.

Given the potential of single-residue polymorphisms to alter ERAP1 activity, combined effects among co-occurring mutations in natural variants are very likely. Thus, to understand the role of natural ERAP1 polymorphism in vivo, the effect of a mutation must be assessed as a function of its structural context. Only recently the effect of natural ERAP1 haplotypes on peptide trimming has been analyzed in live cells (10,18,24). These studies showed that ERAP1 activity depends on the precise combination of amino acid changes in a given variant.

Besides obviating context-dependent effects, previous in vitro studies using single mutants (8,11,12) cannot be directly generalized to HLA-B27 and to the situation in live cells due to some limitations, which we tried to overcome in our study. Since ERAP1 trimming is strongly dependent on the structure of the substrate (13,14,23), extrapolating the effects of ERAP1 polymorphism on other peptides to HLA-B27 ligands is unreliable. Thus, we examined the effect of AS-associated changes on peptide precursors of natural HLA-B27 ligands that were selected for structural diversity, to better assess peptide-dependent effects. Moreover, in vitro conditions, using recombinant enzymes and single substrates, differ significantly from those in human cells, where ERAP1 acts in the presence of ERAP2 (25) and of complex peptide pools that may influence ERAP1 activity (12). These considerations do not invalidate in vitro approaches, but impose a cautious extrapolation and, ideally, a correlation with data from human cells, as done in this study.

The hydrolysis of L-AMC confirmed the lower activity of R528 relative to K528 in a given context, as reported in previous studies (8,11,12), and revealed that N575 conferred higher activity compared to D575. Although the magnitude of this difference was dependent on residue 528, N575 was more active than D575 in both the R528 and K528 contexts. In contrast, the relative activity of R528 and K528 variants was dependent on residue 575, demonstrating the context-dependent effect of these AS-associated mutations in complex allotypes. The induction of higher activity by D575N in the R528 context was independently observed with natural ERAP1 variants and N-terminally extended precursors of an H-2Kb-restricted epitope (18). D575N is so far unique among the AS-protective mutations in that it enhanced ERAP1 activity, since K528R, R725Q and Q730E have the opposite effect.

The relative activity of the four ERAP1 variants towards peptide substrates paralleled that with L-AMC when their joint effects over multiple substrates were considered. However, significant peptide-dependent differences were observed, as expected from the strong dependence of ERAP1 on the structure of the substrate.

We previously reported that AS-associated ERAP1 polymorphism induced quantitative differences in the HLA-B27 peptidome from human cells, affecting many
peptides expressed in distinct ERAP1 contexts (10). Now we found that epitope amounts, which result from the balance between their generation and destruction, were strongly dependent on both ERAP1 activity and digestion time. Frequently, faster generation of the epitope by the most active variant, led to higher yield at shorter times, whereas destructive cleavages tended to equal or decrease epitope yields, relative to less active variants, at long reaction times. This may be relevant to the mechanism of peptide transfer from ERAP1 to the MHC molecule. If ERAP1 would act in close spatial connection with the peptide-loading complex, this could allow a fast transfer of the natural ligand to the MHC, which would protect it from further degradation. This mechanism would be compatible with a protective effect of MHC-I molecules from destructive ERAP-mediated trimming (26), and would enable the most active variants with an advantage to generate many MHC ligands. Alternatively, if ERAP1 generates a peptide pool in the ER that is uncoupled to the peptide-loading mechanism of MHC-I molecules, allowing for a more extensive iteration of substrate trimming, the most active ERAP1 variants would not be necessarily advantageous over less active ones in generating the MHC ligands, as observed in our experiments at long digestion times. This latter alternative explains why a more active ERAP1 variant, with AS-predisposing polymorphisms, reduced the presentation of multiple HLA-B27-restricted ligands, relative to a less active variant expressing multiple AS-protective polymorphisms in a recent study (24). Furthermore, to our knowledge, there is no consistent evidence for any coupling between ERAP1 and the peptide loading-complex of MHC-I that could allow fast peptide transfer to MHC and prevent further epitope degradation.

Given the significant differences between peptide digestion in vitro and in live cells, where many different variables may condition the expression level of HLA-B27 ligands, extrapolating our results to the situation in vivo must be done with great caution. Yet, the close correlation found between the relative yields of RRYQKSTEL and RRYQKSTELLIR both in vitro and in live cells in two distinct ERAP1 contexts, strongly suggests that ERAP1 processing may be a major determinant of the relative amounts of these two ligands in vivo. Of note, that this correlation held only at relatively long digestion times further supports that there is no coordinated peptide transfer from ERAP1 to MHC-I, but rather generation of peptide pools available for MHC-I binding.

That the inhibition of L-AMC hydrolysis by a natural HLA-B27 ligand increased with ERAP1 activity suggests a dominant effect of ERAP1 polymorphism on peptide substrates, relative to small non-peptidic ones. A likely explanation may be that the conformational transition induced by the peptide substrate to the active state of the enzyme favors peptide hydrolysis over that of a small one. Alternatively, the peptide-induced transition to the closed/active conformation may limit access of L-AMC to the catalytic site to an extent that would depend on the efficiency with which the enzyme is activated upon peptide binding.

It has been suggested that small peptides may unproductively bind in the catalytic site, without inducing the transition to the active state, and block the binding of longer, productive substrates (19). Our study showed that the inhibition of the trimming of a peptide substrate by the octamer product of a natural HLA-B27 ligand depended on the activity of the ERAP1 variant. Thus, ERAP1 polymorphism influences the inhibition of peptide processing by short peptides resulting from the destructive cleavage of natural ligands. Although the competition among peptide substrates, as presumably occurs in vivo (12), was not addressed here, our observations show that the influence of ERAP1 polymorphism on peptide trimming has a regulatory component affecting substrate competition, including that mediated by small digestion products.

In conclusion, this study demonstrates that the AS-associated residues 528 and 575 influence ERAP1 function in a mutual context-dependent way, so that the relative activity of variants carrying a given change at one of these positions depends on the polymorphism at the other position. Although for most of the AS-associated polymorphisms the protective alleles (i.e.: R528, Q725, E730) diminish ERAP1 activity, the protective N575 change had the opposite effect. The various residue combinations at both positions affected the generation and destruction of
HLA-B27 ligands in a variant and peptide-dependent way. The resulting effect is both extensive and complex. In some cases both epitope generation and destruction increased similarly with ERAP1 activity, resulting in little alteration in the final yield of the natural ligand at long digestion times. In these cases the ligand was obtained with highest yield by the most active variant at short digestion times, when generation dominated over destruction. In other cases, relative epitope yields among variants were maintained at all times. Our results support that the mechanism of functional interaction between ERAP1 and HLA-B27 involves a widespread alteration in the balance between generation and destruction of HLA-B27 ligands induced by AS-associated ERAP1 polymorphism. This results in large variant- and peptide-dependent effects on epitope levels and, presumably, in the generation or not of particular ligands in a given ERAP1 context, consistent with observations in live cells (10).

Thus, the involvement of ERAP1 in the pathogenesis of AS can be envisaged as a far-reaching influence in the shaping of the HLA-B27 peptidome, affecting peptide binding and presentation in quantitative and qualitative ways, depending on the particular combination of polymorphic residues. As shown here and also noted recently (24) some HLA-B27 epitopes are more extensively destroyed by a more active ERAP1 variant, resulting in their higher production in a less active context. However, other epitopes are predominantly generated in a more active one, as shown here (i.e.: peptides 4 and 8) and in live cells (10).

Besides the obvious effects on antigen presentation, the influence of ERAP1 polymorphism on the peptidome may affect folding and stability of HLA-B27, which also depend on the bound peptides (27). Through its effect on these features, ERAP1 could influence the mechanisms by which HLA-B27 activates the IL23/IL17 axis in spondyloarthropathies, either through misfolding (28) or activation of KIR2DL2+ CD4+ T cells mediated by surface heavy chain homodimers (29), which are generated upon endosomal recycling of HLA-B27 (30). How these features are affected by AS-associated ERAP1 polymorphism is currently a major issue in HLA-B27 research.
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FOOTNOTES

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The abbreviations used are: AS: ankylosing spondylitis; ER: endoplasmic reticulum; L-AMC: Leu-7-amido-4-methyl-coumarine; LD: linkage disequilibrium; MHC-I: Major histocompatibility Complex class I; MS: mass spectrometry; m/z: mass-to-charge; SNP: single nucleotide polymorphism.

FIGURE LEGENDS

FIGURE 1. Hydrolytic activity of ERAP1 variants towards L-AMC. The indicated ERAP1 variants were incubated with the fluorogenic substrate at an E:S ratio 1:30 (w/w) and the fluorescence intensity of the generated fluorofore was measured as a function of time. The data are means of 10 to 13 experiments.

FIGURE 2. Digestion rate of synthetic peptide precursors of HLA-B27 ligands by ERAP1 variants. (A) Combined digestion (%) of peptide species longer than the natural ligand, excluding the original substrate. It was calculated as: 100 - % combined yield of the corresponding species; (B) yield of the natural ligand; (C) yield of peptides shorter than the natural ligand, resulting from destructive cleavages. R528/D575 (−○−), K528/D575 (−■−); R528/N575 (− ■−); K528/N575 (−●−). Yields are relative to the total amount of peptide, which was estimated as the added intensity of the ion peaks corresponding to each peptide species in the MALDI-TOF MS spectrum of the digestion mixture. The data are mean ± s.d. of 3 to 5 experiments. Note that, for any given substrate and time point, the value in panel A is essentially accounted for by the added yield of the corresponding ligand and its digestion product(s) in panels B and C, respectively.

FIGURE 3. Comparison of the digestion of a synthetic peptide substrate analyzed by MALDI-TOF MS and HPLC. (A) Peptide 7 was digested with R528/D575 at the indicated times in parallel experiments and the digestion mixtures were analyzed by MALDI-TOF and HPLC, respectively. Conventions are as in Fig. 2. The left graph shows the combined digestion of peptide species longer than the natural ligand, excluding peptide 7 itself. Digestion was calculated as 100 - % combined yield of the corresponding species. The second and third graphs show the % yield of the natural ligand and of shorter peptide(s), respectively. (B) The same substrate was digested with K528/N575 and analyzed as in A. The data in each panel are from a single experiment, but it was carried out separately for samples to be analyzed by either MS or HPLC.

FIGURE 4. Identification of RRYQKSTELLIR and RRYQKSTEL from C1R-B*27:05 cells. (A) MALDI TOF MS spectra (left) from the HPLC fractions N. 150 and 121, respectively, corresponding to the maximum of the elution profile of each peptide during the fractionation of the B*27:05-bound peptide pool. Only the relevant ion peaks are shown. The sequence of both ligands was determined by MALDI TOF/TOF MS/MS (right); (B) MALDI TOF/TOF MS/MS spectra of the corresponding synthetic peptides.
FIGURE 5. Digestion of two related HLA-B27 ligands of distinct length by ERAP1 variants. The indicated peptides were digested at an E:S ratio 1:10 (w/w) at various times with the following ERAP1 variants: R528/D575 (---■---), K528/D575 (---■---); R528/N575 (- -▲- -); K528/N575 (⋯●⋯). The data are means ± s.d. of 3 experiments.

FIGURE 6. Influence of ERAP1 polymorphism on the inhibition of L-AMC hydrolysis by a natural HLA-B27 ligand. (A) L-AMC was incubated at 100 μM concentration, with the indicated ERAP1 variants at an E:S ratio 1:30 (w/w), in the presence of 100, 75 or 50 μM of RRYQKSTEL for 1 h. The fluorescence intensity of the processed fluorogenic substrate in the absence (black) or in the presence of peptide (white) is indicated. The figures above the histograms indicate the % inhibition of L-AMC hydrolysis. (B) L-AMC was incubated for 1 h at 100 μM concentration, with the indicated ERAP1 variants, at an E:S ratio 1:30 (w/w), in the absence (black) or in the presence (white) of 100 μM of the RYQKSTEL octamer, resulting from the digestion of the nonamer used in panel A. Figures above the histogram bars of the most active variants indicate the % activation of L-AMC hydrolysis. (C) Percent yield of RYQKSTEL after incubation of 100 μM RRYQKSTEL with the indicated ERAP1 variants in exactly the same conditions as in panel A. The data are means ± s.d. of 3 experiments.

FIGURE 7. Influence of ERAP1 polymorphism on the inhibition of peptide trimming by a short peptide. Peptide 6 (LYSELARYGKSPYLY) was incubated with the indicated ERAP1 variants in the presence of various amounts of RYQKSTEL, for 4 h at an E:S ratio 1:10. In each panel, the % yield of the precursor substrate, relative to the total digestion mixture was plotted for the indicated substrate:inhibitor ratios. The figures above the histograms indicate the % inhibition of substrate hydrolysis. The data are means ± s.d. of 3 experiments.
Table I

ERAP1 variants used in this study*  

| SNP     | POLYMORPHISMb | R528/D575 (Wildtype) | K528/D575 | R528/N575 | K528/N575 | C1Rc | WE-Ic |  |
|---------|---------------|----------------------|-----------|-----------|-----------|------|------|  |
| rs26653 | R127P         | R                    | R         | R         | R         | P    | P    |  |
| rs27895 | G346D         | D                    | D         | D         | D         | G    | G    |  |
| rs2287987 | M349V         | M                    | M         | M         | M         | M    | V    |  |
| rs78649652 | G514R         | R                    | R         | R         | R         | G    | G    |  |
| rs30187 | K528R         | R                    | K         | R         | K         | R    | R    |  |
| rs10050860 | D575N         | D                    | D         | N         | N         | D    | N    |  |
| rs17482078 | R725Q         | R                    | R         | R         | R         | R    | Q    |  |
| rs27044 | Q730E         | E                    | E         | E         | E         | E    | E    |  |

*Residues associated with increased risk to AS are underlined. Residues in italics are altered relative to the reference sequence (NCBI accession N.: NP-057526), but have not been reported to influence AS susceptibility. R528/D575 is termed wildtype because this was the starting variant from which the other 3 were generated by site-directed mutagenesis.

bNaturally occurring amino acid changes relative to the reference sequence.

cThe sequence of ERAP1 from these cell lines was previously reported (10).
Table II.

Digestion of peptide precursors by ERAP1 variants

| Peptide                        | R528/D575 | K528/D575 | R528/N575 | K528/N575 | Trimming Score | Mean of F.R. | P1 |
|--------------------------------|-----------|-----------|-----------|-----------|----------------|--------------|----|
| 1 GRHHEAS.IRLPSQYNF            | 15.0 ± 3.0| 15.9 ± 2.5| 34.7 ± 1.1| 79.8 ± 9.2| 1.6            | 2            |    |
| 2 LGVFRKF.SRFPEALRL            | 6.8 ± 1.2 | 36.5 ± 3.4| 76.7 ± 6.9| 95.1 ± 6.7| 1.7            | 2            |    |
| 3 NLKARNS.FRYNGLIHR            | 48.4 ± 5.4| 51.3 ± 2.4| 63.7 ± 1.8| 84.9 ± 1.7| 2.0            | 2            |    |
| 4 IMYKKRT.KRLVVDAR             | 29.6 ± 2.5| 32.8 ± 0.1| 29.9 ± 3.0| 77    ± 2.7| 2.0            | 1            |    |
| 5 DVYAL.KRGRTLYGF              | 10.5 ± 0.7| 3.6 ± 0.5 | 24.6 ± 2.1| 99.9 ± 0.5| 2.5            | 1            |    |
| 6 LYSSEL.ARYKSPYLY             | 79.8 ± 0.3| 72.8 ± 2.3| 79.2 ± 4.1| 77.9 ± 2.6| 2.8            | 4            |    |
| 7 LREI.RRYQKSTEL               | 48 ± 1.2  | 59.3 ± 3.4| 73.7 ± 1.8| 74.5 ± 5.3| 2.0            | 1            |    |
| 8 LREI.RRYQKSTELLIR           | 50.9 ± 5.8| 61.2 ± 2.1| 67.7 ± 0.8| 85.5 ± 1.3| 2.0            | 1            |    |
| MEAN                           | 36.1      | 41.7      | 56.3      | 84.3      |                |              |    |

The data represent the maximal combined digestion (%) of peptide species longer than the natural HLA-B27 ligands (sequences highlighted in boldface) resulting from digestion of the synthetic precursor by the indicated ERAP1 variant. It was calculated as: 100 - % combined yield of the corresponding species. The minimal and maximal values observed with the peptides tested with each variant are highlighted in boldface. The fully processed natural ligands RRYQKSTEL (peptide 9) and RRYQKSTELLIR (peptide 10) are not included. The data are means ± s.d. of at least 3 experiments.

Trimming susceptibility scores for each residue are based on relative cleavage efficiencies as determined in a previous study (13): score 1: 0-25% (T,H,Q,G,N,E,W,D,K,V,R,P); 2: 25-50% (F, I, S); 3: 50-75% (C); 4: 75-100% (A, L,M,Y). For each substrate the mean score of all the flanking residues (Mean of F.R.) and the score of the N-terminal residue of the natural ligand (P1) are shown.
Table III

HLA-B27 epitope destruction by ERAP1 variants

| Peptide | R528/D575 % Max | K528/D575 % Max | R528/N575 % Max | K528/N575 % Max |
|---------|----------------|-----------------|----------------|----------------|
| 1 GRHHEAS.IRLPSQYNF | 6.9 ± 0.4 | 9.1 ± 0.9 | 14.4 ± 1.1 | 78.1 ± 9.2 |
| 2 LGVFRKF.SRFPEALRL | 0.3 ± 0.1 | 5.6 ± 1.2 | 67.5 ± 6.9 | 82.6 ± 6.7 |
| 3 NLKARNS.FRYGLIHR | 23.1 ± 2.7 | 25.7 ± 0.8 | 46.4 ± 1.8 | 81.7 ± 1.7 |
| 4 IMYKRT.KRLVFDAR | 21.8 ± 2.5 | 27.5 ± 0.1 | 22.4 ± 3.0 | 66.6 ± 2.7 |
| 5 DVYYAL.KQGRTLYGF | 10.5 ± 0.7 | 3.6 ± 0.5 | 22.8 ± 2.1 | 99.9 ± 0.5 |
| 6 LYSESL.ARYGKSPYLY | 58.5 ± 2.6 | 71.5 ± 2.3 | 78.4 ± 4.1 | 76.6 ± 2.6 |
| 7 LREI.RRYQKSTEL | 14.1 ± 0.3 | 17 ± 3.5 | 23 ± 2.2 | 44.5 ± 2.6 |
| 8 LREI.RRYQKSTELLIR | 44.5 ± 3.1 | 54.2 ± 2.1 | 62.2 ± 0.8 | 76.8 ± 1.3 |
| **MEAN** | **22.5** | **26.8** | **42.1** | **75.9** |

The data represent the maximal % yield of peptide species shorter than the natural HLA-B27 ligands (sequences highlighted in boldface) by the indicated ERAP1 variant. For each variant the minimal and maximal values observed with the peptides tested are highlighted in boldface. Peptides 9 (RRYQKSTEL) and 10 (RRYQKSTELLIR) are not included. The data are means ± s.d. of at least 3 experiments.
Table IV

HLA-B27 epitope generation by ERAP1 variants

Detection indicates the time (min) and % yield where the HLA-B27 ligand was detected at ≥ 1% of the total digestion mixture with the indicated ERAP1 variant. Since peptide 5 did not reach this value with R528/D575 the longest digestion time was assigned. Maximum indicates the earliest time (min) and the % yield where the HLA-B27 ligand was detected at ≥80% of the maximal value observed with the indicated ERAP1 variant. The data are means ± s.d. of at least 3 experiments.

| Peptide          | R528/D575 |          | K528/D575 |          | R528/N575 |          | K528/N575 |          |
|------------------|-----------|----------|-----------|----------|-----------|----------|-----------|----------|
|                  | Detection | Maximum  | Detection | Maximum  | Detection | Maximum  | Detection | Maximum  |
|                  | Min %     | Min %    | Min %     | Min %    | Min %     | Min %    | Min %     | Min %    |
| 1 GRHHEAS. IRLPSQYNF | 5 1 ± 0.1 | 120 6.6 ± 1 | 5 2.1 ± 0.2 | 60 6.7 ± 0.9 | 15 2 ± 0.1 | 30 9.2 ± 1.9 | 5 9.6 ± 0.3 | 15 13 ± 1.2 |
| 2 LGVFRKF. SRFPEALRL | 180 3.5 ± 0.2 | 240 6.4 ± 0.8 | 180 1.3 ± 0.2 | 240 22.3 ± 3.5 | 30 3.3 ± 1 | 180 4.25 ± 2.4 | 15 1.3 ± 0.5 | 120 48 ± 0.6 |
| 3 NLKARN. FRYNGLIHR | 5 4.4 ± 0.4 | 30 24.7 ± 3.5 | 5 11.5 ± 0.2 | 15 22.8 ± 2.0 | 5 5.5 ± 2.3 | 15 26.7 ± 3.1 | 5 34 ± 2.7 | 5 34 ± 2.7 |
| 4 IMYKRT. KRLVFDBAR | 15 3.2 ± 0.2 | 120 5.3 ± 0.5 | 5 3.7 ± 0.6 | 60 5 ± 0.7 | 15 2.2 ± 0.2 | 180 6.5 ± 0.1 | 15 6.1 ± 0.6 | 60 16.3 ± 0.5 |
| 5 DVYAL. KRQGRTLYGF | 240 0.4 ± 0.1 | 60 0.4 ± 0.1 | 180 1.2 ± 0.1 | 180 1.2 ± 0.1 | 30 1.1 ± 0.1 | 120 1.6 ± 0.1 | 30 1.8 ± 0.2 | 60 2.6 ± 0.1 |
| 6 LYESL. ARYKSPYLY | 5 2.7 ± 0.3 | 60 28.7 ± 0.4 | 5 1.9 ± 0.3 | 15 3 ± 0.9 | 5 2 ± 0.1 | 5 2 ± 0.1 | 5 2 ± 0.1 | 5 2 ± 0.1 |
| 7 LREI. RRYQKSTEL | 5 1 ± 0.1 | 180 32.7 ± 0.2 | 5 1.1 ± 0.5 | 120 33 ± 2.8 | 5 3.1 ± 0.1 | 120 49.8 ± 2.8 | 5 27.8 ± 0.2 | 5 27.8 ± 0.2 |
| 8 LREI. RRYQKSTELLIR | 5 6.9 ± 0.4 | 5 6.9 ± 0.4 | 5 3.3 ± 1 | 60 5.6 ± 1.2 | 5 5.2 ± 0.2 | 5 5.2 ± 0.2 | 5 7.2 ± 1.2 | 15 9.9 ± 1.4 |
| MEAN             | 55.7 2.9 | 101.9 14.0 | 48.8 3.3 | 93.8 12.5 | 13.8 3.1 | 93.1 17.9 | 10.6 11.2 | 35.6 19.2 |

*aDetection indicates the time (min) and % yield where the HLA-B27 ligand was detected at ≥ 1% of the total digestion mixture with the indicated ERAP1 variant. Since peptide 5 did not reach this value with R528/D575 the longest digestion time was assigned. Maximum indicates the earliest time (min) and the % yield where the HLA-B27 ligand was detected at ≥80% of the maximal value observed with the indicated ERAP1 variant. The data are means ± s.d. of at least 3 experiments.*
Table V

Generation of length variants of HLA-B27 ligands in vitro and in live cells

| % Yield from their precursors in vitro<sup>a</sup> |
|-----------------------------------------------|
| R528/D575 | R528/N575 |
|-----------|-----------|
| Min | RRYQKSTEL | RRYQKSTELLIR | Ratio | RRYQKSTEL | RRYQKSTELLIR | Ratio |
| 5  | 1.0 ± 0.1 | 6.9 ± 0.1 | 0.1 | 3.1 ± 0.2 | 5.2 ± 0.1 | 0.6 |
| 15 | 3.5 ± 0.1 | 5.3 ± 1.6 | 0.7 | 4.8 ± 0.2 | 6.1 ± 2.1 | 0.8 |
| 30 | 7.7 ± 1.0 | 4.9 ± 1.7 | 1.6 | 16.9 ± 0.7 | 5.2 ± 2.0 | 3.3 |
| 60 | 23.7 ± 0.2 | 5.2 ± 2.0 | 4.5 | 39.2 ± 2.3 | 6.0 ± 1.0 | 6.6 |
| 120| 22.4 ± 2.1 | 4.9 ± 1.2 | 4.6 | 49.6 ± 3.7 | 5.1 ± 0.5 | 9.7 |
| 180| 32.7 ± 0.5 | 6.4 ± 0.8 | 5.1 | 52.0 ± 0.6 | 5.1 ± 1.4 | 10.2 |
| 240| 35.2 ± 3.0 | 4.8 ± 1.0 | 7.3 | 48.4 ± 2.2 | 5.6 ± 1.3 | 8.7 |

| Human cells | Intensity of ion peak<sup>b</sup> |
|--------------|----------------|
| Cell line    | RRYQKSTEL | RRYQKSTELLIR | Ratio |
| WEWAK I (RN) | 31350     | 4711         | 6.7   |
| C1R-04 (RD)  | 42867     | 10258        | 4.2   |
| C1R-05 (RD)  | 62232     | 8405         | 7.4   |

<sup>a</sup>The data are from the digestions of peptides 7 and 8 at the indicated times (Fig. 2B) and are means ± s.d. from 3 (RRYQKSTELLIR) or 5 (RRYQKSTEL) independent experiments.

<sup>b</sup>The data are means from 4 (Wewak I), 8 (C1R-04) and 3 (C1R-05) independent preparations, respectively.
Figure 1
Figure 3
Figure 5

[Graph showing % yield over time for two different peptides: RRYQKSTEL and RRYQKSTELLIR.]
Figure 6

A

Fluorescence intensity

B

Fluorescence intensity

C

% Yield

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Figure 7
Combined effects of ankylosing spondylitis-associated ERAP1 polymorphisms outside the catalytic and peptide-binding sites on the processing of natural HLA-B27 ligands

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