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A Common Single Nucleotide Polymorphism in Endoplasmic Reticulum Aminopeptidase 2 Induces a Specificity Switch That Leads to Altered Antigen Processing

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Endoplasmic reticulum aminopeptidases 1 and 2 (ERAP1 and ERAP2) cooperate to trim antigenic peptide precursors for loading onto MHC class I molecules and help regulate the adaptive immune response. Common coding single nucleotide polymorphisms in ERAP1 and ERAP2 have been linked with predisposition to human diseases ranging from viral and bacterial infections to autoimmunity and cancer. It has been hypothesized that altered Ag processing by these enzymes is a causal link to disease etiology, but the molecular mechanisms are obscure. We report in this article that the common ERAP2 single nucleotide polymorphism rs2549782 that codes for amino acid variation N392K leads to alterations in both the activity and the specificity of the enzyme. Specifically, the 392N allele excises hydrophobic N-terminal residues from epitope precursors up to 165-fold faster compared with the 392K allele, although both alleles are very similar in excising positively charged N-terminal amino acids. These effects are primarily due to changes in the catalytic turnover rate (kcat) and not in the affinity for the substrate. X-ray crystallographic analysis of the ERAP2 392K allele suggests that the polymorphism interferes with the stabilization of the N terminus of the peptide both directly and indirectly through interactions with key residues participating in catalysis. This specificity switch allows the 392N allele of ERAP2 to supplement ERAP1 activity for the removal of hydrophobic N-terminal residues. Our results provide mechanistic insight to the association of this ERAP2 polymorphism with disease and support the idea that polymorphic variation in Ag processing enzymes constitutes a component of immune response variability in humans. The Journal of Immunology, 2012, 189: 000–000.

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I.E. performed the biochemical characterization of the variants, designed experiments, and interpreted data. J.B. generated the ERAP2K variant and crystallized the protein. S.S. and A.A. designed and performed the cell-based assay. A.P. performed modeling and computational analysis. E.Z. purified and characterized the inhibitor. M.S. and G.P. designed and performed the mass-spectrometric analysis. P.G. collected and processed the X-ray diffraction data. O.P. and D.G. designed and synthesized the inhibitor. E. Saridakis, J.B., and I.M.M. solved and interpreted the crystal structure. E. Stratikos conceived the project, designed experiments, interpreted results, and wrote the manuscript together with input from other authors.

Atomic coordinates and structure factors have been deposited in The Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under Protein Data Bank ID 4E36.

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Abbreviations used in this article: ER, endoplasmic reticulum; ERAP, endoplasmic aminopeptidase; kcat, catalytic turnover rate; MS, mass spectrometry; R-AMC, L-arginyl-7-amido-4-methyl coumarin; RP-HPLC, reverse phase HPLC; SNP, single nucleotide polymorphism; SR, specificity ratio; TFA, trifluoroacetic acid.

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aminopeptidase, insulin-regulated aminopeptidase, has been recently implicated in a separate endosomal pathway of cross-presentation in specialized cells (4). Of these three, the better characterized one, ERAP1 has been shown to play the role of an antigenic peptide editor, influencing the antigenic peptide repertoire and the adaptive immune response in vivo (6, 10–12). ERAP1 achieves peptide editing by both generating many mature epitopes from elongated precursors and by destroying others by further trimming them to smaller peptides that cannot bind onto MHC class I molecules (8, 13). Less is known about the function of ERAP2, although it has been proposed that it acts as a necessary aminopeptidase, complementing ERAP1 specificity to allow the trimming of the vast number of different sequences in the antigenic peptide repertoire (9, 14, 15). ERAP1 has some key functional properties that make it suitable for antigenic peptide precursor trimming: it is specialized for relatively large peptides and can trim peptides depending on their length and internal sequence (16, 17). Furthermore, ERAP1 can be regulated by its own substrates and products, suggesting a complex and poorly understood landscape of the regulation of Ag processing (18, 19). ERAP2 and insulin-regulated aminopeptidase, although less studied to date, appear to share some of those properties, but also have key differences that may underlie distinct biological roles (9, 13, 15, 16).

Several large-scale, population-wide genetic studies have linked specific amino acid coding single nucleotide polymorphisms (SNPs) in ERAP1 and ERAP2 with predisposition to human disease (reviewed in Ref. 20). SNPs in ERAP1 and ERAP2 have been linked with predisposition for development of the chronic inflammatory disease ankylosing spondylitis, a disease with a strong hereditary autoimmune component (21–23). The original hypothesis that this link is mediated by the Ag processing function of ERAP1/ERAP2 was later supported by the demonstration of interplay between ERAP1 and ERAP2 SNPs and more importantly with the MHC class I allele HLA-B27 (24–26). Recently, ERAP1/ERAP2 haplotypes have been linked with resistance to HIV infection (27). More specifically, the ERAP2 SNP rs2549782 that codes for the variation N392K has been linked with resistance to HIV infection in homoygous individuals and with development of pre-eclampsia (27–29). ERAP1 and ERAP2 polymorphisms have been proposed to be the result of a long-standing balancing selection, indicating significant functional consequences of both polymorphic states (27, 30). Preliminary results regarding the effects of coding ERAP1 polymorphisms have pointed toward changes in both enzyme activity and specificity that may influence the antigenic peptide repertoire (19, 26, 31). Although these changes in antigenic peptide processing have been of a relatively low magnitude (generally up to 2-fold), it has been hypothesized that they can be sufficient in altering chronic immune and/or inflammatory responses (19, 26).

In this study, we characterized the ERAP2 SNP rs2549782 that codes for the amino acid variation N392K. This particular polymorphism constitutes an attractive model for understanding the role of polymorphic variation in Ag processing for three reasons: 1) both polymorphic states are almost equally distributed in the human population, presumably as a result of host–pathogen balancing selection (27); 2) it is the main polymorphism in ERAP2 that has been associated with disease in virtually all genetic studies; and 3) its structural proximity to the enzyme’s active site makes it reasonable to expect at least some perturbation in enzymatic activity (15).

We used a combination of biochemical, cell-based, structural, and computational approaches to understand the effects of this polymorphic variation to ERAP2 function. We demonstrate that the polymorphic variation at position 392 affects both the activity and specificity of the enzyme up to two orders of magnitude, altering its Ag processing profile, as well as its ability to complement ERAP1-mediated Ag processing. Structural and biochemical analysis suggests that these effects are due to changes in the catalytic site of the enzyme that affect transition-state stabilization. To our knowledge, these are the largest functional changes described for a common coding polymorphism in the Ag processing machinery. Our results provide mechanistic insight to the hypothesized causal link between this ERAP2 SNP and disease. Furthermore, our results support the hypothesis that allelic variability in Ag processing enzymes is an important component of immune system variability in natural populations.

### Materials and Methods

#### Peptides

Peptides WLRRYLENGK, RLRRYLENGK, LSLYNTVAL, KSLYNTVAL, LSRHHAFSFR, RSRYYWAIRTR, LSRYWAIRTR, KSRYYWAIRTR, YKRFEQITQOR, LKRYVINKDT, and ASRHHAFSFR were purchased from JPT Peptide Technologies (Berlin, Germany). Peptide KSLYNTVAL was purchased from Invitrogen. All peptides were purified by reverse phase HPLC (RP-HPLC) and were >95% pure.

#### Protein expression and purification

Construction of the N392K ERAP2 variant (ERAP2K) was performed using the Quikchange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) on the pFastbac1–ERAP2K vector (described in Ref. 14). The primers used for the variant construction were: 5′–GAG AGT ATC TTC GCC TTA AGG AGG GTT TTG CAA AAT AC-3′ (sense) and 5′–GTA TTT TGC AAA ACC CTC AAG CCA GAT ATC ATT CC-3′ (antisense). DNA sequencing was performed to validate successful mutagenesis.

Overexpression of ERAP2K in insect cell cultures has been described previously (14, 15). The pFastbac1–ERAP2K vector was used to generate recombinant baculovirus according to the directions of the bac-to-bac baculovirus expression system (Invitrogen), as described before (17). Human recombinant ERAP2K was produced in HEK cells postinfection with recombinant baculovirus carrying the ERAP2 gene with a C-terminal 6-His tag. The cell supernatant was harvested 4 d postinfection by centrifugation and subjected to Ni-NTA affinity chromatography purification as described previously (15, 17). In brief, after binding in the presence of the bacterial form of the enzyme containing 1 mM imidazole for 2 h, protein was eluted from the column using step gradient of 3, 5, 10, 20, 40, and 150 mM imidazole solutions (Supplemental Fig. 1A). The fractions with highest enzymatic activity and purity were collected, dialyzed to remove the imidazole, and stored at −80°C in aliquots in the presence of 10% glycerol, until needed. Each aliquot was thawed once, used in assays immediately, and then discarded. Both ERAP2 variants were overexpressed and purified in parallel for purposes of comparing enzymatic activities.

#### Enzymatic assays

The activity of human recombinant ERAP2 was measured by following the time-dependent increase of the fluorescence signal at 460 nm of the fluorogenic substrate L-arginyl-7-amido-4-methyl coumarin (R-AMC; Sigma). Excitation was set at 380 nm. Fluorescence was followed for 5 min using a QuantaMaster–4 fluorometer (Photon Technology International, Lawrenceville, NJ), and the resulting time slope was used to calculate the rate of hydrolysis of the substrate. ERAP2 specificity for small fluorogenic substrates was analyzed using a library of fluorogenic compounds described before (14, 32). A total of 10 μM of each substrate was incubated with 8 ng ERAP2 (total volume, 140 μl; ERAP2 concentration, 0.52 nM) at 25°C, and fluorescence was recorded for 5 min with excitation set at 380 nm and emission at 460 nm using a Tecan Infinite M200 microplate fluorescence reader. Inhibition data were fit to a simple binding model 

\[ V = V_o \times X / (K_i + X) - V_i \]

where \( V\) is the rate of hydrolysis in the absence of inhibitor. Inhibition by the DG001 peptide was found to proceed to <100%, and data were fit to a partial inhibition model 

\[ V = V_o \times X / (K_i + X) - V_i - B, \]

in which the parameter \( B\) is the maximal inhibition attained after saturation.

#### Synthesis, purification, and characterization of pseudopeptide transition-state analogs

The L-[[PO(OH)CH2]-LAFKARAF peptide (DG001) was synthesized by Fmoc-solid–phase peptide synthesis using the multipin technology as
previously described (33). Peptide Fmoc-AFKARAF was developed on triyl alcohol lanterns (surface: polyurethane, loading 15 μmol) using the Fmoc-solid−phase protocol (34). Pmc and Boc protecting groups were used for the side-chain functional groups of Arg and Lys, respectively. After the deprotection of the N-terminal terminal, the phosphinic dipeptide unit was introduced by shaking the lantern-anchored peptides with a solution of 1.3 equivalent of phosphonic block Boc-Leu-Ψ(PPO−Oad−CH2)3−LeuOH, DIC (2 equivalents), and HOBr (2 equivalents) in dichloromethane during 24 h. The phosphonic block was synthesized based on a previously described protocol (35). The crude peptide was obtained after treatment of lanterns with trifluoroacetic acid (TFA)−dicloromethane/H2O/Ottrisopropylsilane 80/15/2.5:2.5 for 2 h, and purified by RP-HPLC using a Chromolith C-18 semiprep column (Merck) and a 5−50% acetonitrile gradient in water containing 0.05% trifluoroacetic acid. Eluted peaks were collected, lyophilized, and characterized by mass spectrometry (MS).

Analysis of peptide trimming activity on RP-HPLC

Analysis of digestion products after incubation of peptides with ERAP1 or ERAP2 was performed by RP-HPLC on a Chromolith C-18 analytical column (Merck) as previously described (17). In brief, 20 μL of each peptide was incubated at 37 °C with 100−400 ng (7.3−293.6 nM) ERAP1 or ERAP2 in 50 mM HEPES pH 7.0, 100 mM NaCl buffer in a total volume of 125 μL for 15 min to 1 h. After incubation, the reactions were terminated by adding 100 μL of 1% trifluoroacetic acid and stored at −20 °C until analysis. Before analysis, samples were centrifuged for 5 min at 10,000 g to remove precipitated protein. HPLC elution was performed using a 5−50% acetonitrile gradient, while following the absorbance at 220 nm. To calculate initial reaction rates, we performed several experiments for each peptide to achieve conditions under which the reaction was <30% complete. All comparisons of the activity of ERAP1 and the two ERAP2 variants were performed in parallel. Product peaks in the HPLC chromatogram were identified by running peptide controls and by MS as described previously (13).

Analysis of peptide trimming activity by mass spectroscopy

Trimming of ASRHHAFSFR peptide by ERAP2 was followed by mass spectroscopy because the product SHRHAFSFR is poorly separated by HPLC. Reaction mixtures were set up as described earlier with the exception that they were stopped by adding 1% formic acid. The reactions were initially cleaned up using pipette tips packaged with reverse-phase material (C18MB, OMIX, VARIAN). The samples were dried completely in a speed-vac, dissolved in 100 μL of 0.1% formic acid and 2% acetonitrile, and sonicated for 1 min. The samples were analyzed by direct infusion nano-electrospray on an LTQ Orbitrap XL mass spectrometer at a resolving power of 60,000 at m/z 400. The scan range was set to 350−550 μz. MS/MS spectrum was acquired to confirm the sequence of the peptides. The triply charged peptides were monitored. Reaction progress was evaluated by measuring the relative peak intensity of the substrate and product peptide, respectively. All reactions were evaluated at >30% completion to ensure linearity of the rate measurement.

Cell-based Ag presentation assay

Construction of plasmids, encoding for ERAP2N and ERAP2K variants (rs2549782, GenBank accession no. NM_022350.3), was performed as previously described for ERAP1 alleles (7, 19) in pTracerCMV-RFP, where the GFP/Zeo fusion protein was replaced with red fluorescent protein ORF. In brief, ERAP2N ORF was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). pcDNA-ERAP2N was generated using Quikchange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), using the primers: ERAP2 Start_SEQ (reverse), ERAP2MUT (forward), 5’-GGAGATATGATGCTGACAGGAGGTGGTTTGGGTCAATACTAC-3’; ERAP2_MUT (reverse), 5’-GTATTTGAGAAACCCCTGCTAAAGGCGCTATTCATCCC-3’. pcDNA-based ERAP2 containing plasmids were digested with SalI and XhoI, and subcloned into pShuttleCMV pShuttle-ERAP2 plasmids were digested with NotI and XbaI, and subcloned into pTracerCMV-RFP. In- tegron ERAP2 allele density close to the gene of the active site of each plasmid was confirmed by sequencing of whole-length ERAP2 ORF using the following primers: ERAP2P1_seq (forward), 5’-AAGATTGGCACTGTGCTTGCATCCTTCTATGGC-3’; ERAP2P1_Seq (reverse), 5’-CTCAATTCGTGAGGCGGAGATGCTAGGCGGAAATG-3’; ERAP2P2_seq (forward), 5’-CAACCCAGCCAGCGACCCTGG-3’. pcDNA-based ERAP2 were transiently transfected into HeLa cells, stably expressing H-2Kb, HLA-B27, and Herpesvirus protein ICP47 (TAP1 blocker). HeLaK-B2747 were described previously (8). To construct plasmids expressing HLA-B27-restricted peptides (and their N-terminal precursors), we subcloned nucleotide sequences corresponding to ASRHHAFSFR and SHRHAFSFR peptides into pTracerCMV (Invitrogen, Carlsbad, CA). All peptides were designed to contain the ER-signaling sequence MRYMLGLAALAVCSA (from the Ad2E3gp19K protein) upstream of the peptide sequence (7). HeLaK-B2747 were transiently transfected with HLA-B27−specific peptide and either ERAP2N or ERAP2K−expressing plasmids for 48 h. Surface expression of HLA-B27 in transfected cells was measured by flow cytometry, exactly as described previously (19). Statistical analysis was completed using one sample t test (Fmean ratio − 1)/SE to test whether Ag presentation in ERAP2 transfected cells deviated from mock transfections performed in parallel.

Quantitation of protein expression in HeLa cells

HeLaK-B2747 cells were transfected with pTracer-CMV-RFP-ERAP2N (or ERAP2K). At 24 h after transfection, protein was harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 with protease inhibitors. Lysed samples were then centrifuged at maximum speed (16,000 × g) for 15 min at 4 °C, after which the supernatant was collected. The lysates were then subjected to Western blotting with anti-HLA-B27 (anti-B7) or anti-HLA-A2 (anti-2B7) monoclonal antibodies (BD Biosciences). The bands were quantified using Licor’s Odyssey scanner (models 2.1 or 3.0). For data analysis, the fluorescence of ERAP2 bands was normalized to Tubulin bands before quantification.

X-ray crystallography

Crystalization of ERAP2 was performed using conditions identical to the ones described previously for ERAP2N (15). During the last stages of concentration, DG001 peptide was added in the protein solution at a protein/peptide ratio of 1:4; the mixture was incubated at room temperature for 4 h, and the sample was further concentrated to 6 mg/mL. Single crystals were obtained as for ERAP2N using the Morpheus (36) protein crystallization screen (Molecular Dimensions, Newmarket, U.K.) by the sitting drop vapor diffusion technique: by mixing 200 nl ERAP2N (6 mg/mL in 150 mM NaCl, 0.01% Na2, 25 mM HEPES pH 7.0) with 100 nl precipitant using an Oryx4 nano-drop crystallization robot and incubating against 50−μl reservoir. Initial crystals appeared after 2−3 d. The crystal was obtained for data collection was grown in a 2-μl drop at 4 °C, using the condition 10% PEG 8000, 20% ethylene glycol, 20 mM each of glycine, dl-alanine, dl-serine, dl-lysine, and Na-glutamate, 31 mM MES, and 69 mM imidazole at pH 6.5. X-ray diffraction data were collected at 100 K using synchrotron radiation at the X06DA beamline (Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland). Diffraction data up to 2.2 Å resolution were processed by MOSFILM (37) and scaled with the SCALSA software (38). Five percent of reflections was flagged for Rfree calculations. The crystal is isomorphous with that of ERAP2N, space group P21, a = 74.3 Å, b = 134.4 Å, c = 127.4 Å, and β = 90.8° (Supplemental Table I). The structure was determined by the isomorphous replacement method using the ERAP2N coordinates (PDB Databank ID is 3E6F). Phenix using BCA method. Equivalent concentrations of protein samples (5−20 μg) were run on 8% polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were then probed with the mouse anti-αTubulin monoclonal primary Ab (Sigma-Aldrich, St. Louis, MO) at 1:50,000 dilution for 20 min and primary polyclonal mouse-anti-human ERAP2 (Abcam, Cambridge, MA) at 1:1000 dilution for 3−16 h. Membranes were washed with 5% nonfat milk/fluorescent Abs (IRDYE800 conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA) for 45 min at 1:3300 dilution. Blots were scanned and bands were quantified using Licor’s Odyssey scanner (models 2.1 or 3.0). For data analysis, the fluorescence of ERAP2 bands was normalized to Tubulin bands before quantification.
To investigate whether the change in catalytic turnover was substrate specific, we used a previously characterized collection of fluorogenic substrates bearing all-natural amino acids as their scissile N termini (14, 32). Consistent with previous observations on the specificity of ERAP2, both polymorphic states presented a highly similar pattern of N-terminal selectivity with only positively charged side chains processed efficiently (Supplemental Fig. 1B). This finding suggests that the N392K polymorphism affects the catalytic efficiency but not the specificity of ERAP2, at least toward these small fluorogenic substrates.

Trimming of antigenic peptide precursors

Because the trimming of small fluorogenic substrates does not adequately describe the in vivo function of the enzyme, we tested the trimming of antigenic peptide precursors by the two ERAP2 alleles. ERAP2N was able to efficiently trim the leucine residue from the precursor peptide with the sequence LSRHHAFSFR and generate the mature epitope SRHHAFSFR (from the aggresome protein, a critical component for cartilage structure and function of the joints, normally presented by the HLA-B2705 MHC class I allele; Fig. 2A). This result was rather surprising given the postulated role of ERAP2 in removing positively charged amino acids (9, 14) and may be because of other factors including the recognition of side chains distal from the N terminus allowing the processing of peptides with suboptimal N termini, a property established for ERAP1 (19) (also see later). In contrast with ERAP2N, ERAP2K was much less efficient in processing this antigenic peptide precursor and generated the mature epitope with a 37-fold slower rate (Fig. 2A, 2B). This change in trimming rate was not due to substrate recognition because the $K_M$ for this peptide was similar for both alleles (33 ± 9 versus 19 ± 5 μM) as calculated by competition titrations (Fig. 2C). We conclude that the difference in peptide processing kinetics is due to a change in the $k_{cat}$ of the enzyme for this particular substrate (Fig. 2D).

ERAP2 has been described previously as an accessory aminopeptidase that “assists” ERAP1 in trimming positively charged residues from antigenic peptide precursors, residues for which ERAP1 has reduced activity (9, 14). To test whether ERAP2 allelic variation affects the trimming of a peptide precursor with a positively charged N terminus, we measured the kinetics of trimming of a peptide with the sequence KSLLNYTVATL, a precursor to the epitope SLYNTVATL (derived from the HIV-1 p17 Gag protein and presented by the HLA-A0201 MHC class I allele). In sharp contrast with the SRHHAFSFR epitope, SLYNTVATL was produced equally effectively by both ERAP2 alleles (Fig. 3). This surprising result suggested that the change in $k_{cat}$ between ERAP2N and ERAP2K may be substrate dependent for larger, more physiologically relevant, peptidic substrates.

To test the generality of changes in substrate specificity between the two ERAP2 alleles, we measured the trimming rates for eight additional antigenic peptide precursors in vitro. These precursors were designed based on the protein sequence upstream of antigenic peptides that are known to be generated by the action of intracellular aminopeptidases. The N-terminal amino acid was in every case either a positively charged residue or a hydrophobic residue. The trimming rates for all precursors tested are compared in Fig. 4. In most cases, there was a significant difference between the trimming rates for the two alleles; these differences were much more potent for precursors with a hydrophobic N-terminal amino acid. For example, the precursors RSRYWAIRTR and KSLYNTVATL were processed with almost identical rates by both ERAP2 alleles. In contrast, the precursor WRLRYLENKG was processed with a rate difference of 165-fold (330 ± 34 pmol nmol$^{-1}$ sec$^{-1}$ for ERAP2N versus 2.0 ± 0.04 pmol nmol$^{-1}$ sec$^{-1}$ for ERAP2K).

**Results**

**ERAP2K** is less active in hydrolyzing small fluorogenic substrates

Small fluorogenic substrates such as R-AMC have been used previously to characterize the activity and specificity of M1 aminopeptidases (14). To compare the enzymatic activity of the two ERAP2 variants, we measured the rate of hydrolysis of R-AMC by ERAP2N and ERAP2K. The two variants had significantly different activity, with R-AMC being hydrolyzed at 78 ± 1 mmol product/mmol enzyme sec$^{-1}$ by ERAP2N and 4.8 ± 3 mmol/ mmol sec$^{-1}$ by ERAP2K. Such a difference in activity is at least 10-fold higher compared with previously characterized ERAP1 polymorphisms (19, 31). To investigate whether this change in catalytic activity was due to changes in substrate recognition or catalytic turnover rate ($k_{cat}$), we performed a standard Michaelis–Menten analysis (Fig. 1). According to this analysis, the polymorphic state at position 392 of ERAP2 affected substrate affinity by $<2$-fold (ERAP2N: $K_M = 30 ± 9$ μM; ERAP2K: $K_M = 17 ± 5$ μM) but affected $k_{cat}$ to a large degree, lowering the $k_{cat}$ constant by $–18$-fold (ERAP2N: $k_{cat} = 104 ± 9 × 10^{-3}$ sec$^{-1}$; ERAP2K: $k_{cat} = 5.8 ± 0.4 × 10^{-3}$ sec$^{-1}$). This large change in catalytic efficiency brought about by a common polymorphism is, to our knowledge, the largest described for this class of enzymes to date.

**FIGURE 1.** (A) Michaelis–Menten analysis of R-AMC hydrolysis by ERAP2N (filled circles) and ERAP2K (open circles) variants. (B) Double-reciprocal plot of the data.
We concluded from this limited screen that the polymorphic variation in position 392 of ERAP2 can affect peptide specificity, primarily for peptides that carry a hydrophobic amino acid as their N terminus. This is in sharp contrast with what we discovered for small nonpeptidic substrates (Fig. 1, Supplemental Fig. 1), in which case, we could only demonstrate a difference of catalytic turnover between the two alleles but not in specificity. This apparent paradox may signify fundamental differences between recognition of small versus larger natural substrates. Such a distinction has recently been described for ERAP1 and has been hypothesized to constitute the main mechanism for efficient large peptide trimming necessary for its biological function (18).

In an effort to isolate the importance of the N-terminal residue of the peptide substrate in the trimming rate by the two ERAP2 alleles, we compared the trimming of identical peptide backbones that only differ in the N-terminal residue. The ratio of the trimming rate of the peptide carrying an N-terminal extension of a positively charged amino acid versus the trimming rate of the same peptide carrying a hydrophobic N-terminal extension defines a specificity ratio (SR) that indicates the preference of ERAP2 for positive versus hydrophobic N termini. The calculated SR value for three antigenic epitopes is shown in Fig. 5A. In all cases, ERAP2K showed a clear preference for positively charged residues at the N terminus of the peptide (SR > 1, maximal SR = 92), although the magnitude of this value depended on the remaining peptidic sequence, indicating that the whole peptide sequence is important for substrate recognition (as has been demonstrated for ERAP1; see Ref. 17). In addition, in all cases, the SR value for ERAP2N was significantly lower, indicating that the strong preference for positively charged N termini is not shared by this allele that can efficiently process both hydrophobic and positively charged N termini. These differences in specificity are highly significant and can lead to qualitative differences in trimming between the two alleles. As shown in Fig. 5B, trimming of the precursor RSRYWAIRTR is essentially identical for both alleles, whereas trimming of the precursor LSRYWAIRTR (same sequence but with different N terminus) is drastically different, with ERAP2K essentially failing to produce detectable amounts of mature epitope under the specific experimental conditions. Overall, these results suggest that the polymorphism at position 392 of ERAP2 can induce changes in specificity when trimming natural peptidic substrates.
ERAP2N can supplement ERAP1-mediated trimming of hydrophobic N termini

ERAP2 has been proposed to act as an accessory aminopeptidase to ERAP1, assisting it in trimming peptide sequences for which ERAP1 has suboptimal affinity such as peptide carrying positive-charged N-terminal amino acids (9). Our finding that ERAP2N can excise hydrophobic N termini much more efficiently than ERAP2K prompted us to investigate how well ERAP2 activity compares with ERAP1. We measured the rate of generation of the SRHHAFSFR epitope from a precursor carrying an N-terminal leucine residue, by ERAP1, ERAP2N, and ERAP2K (Fig. 6A). Because ERAP1 has been reported to also be able to destroy antigenic epitopes, we measured the rate of destruction of the same epitope by quantifying smaller peptides accumulated after treating it with each of the three enzymes (characteristic chromatograms of epitope generation and destruction are shown in Supplemental Fig. 2). All three enzymes generated the epitope faster than they destroyed it, suggesting that they have the inherent ability to accumulate it (13). This effect was more pronounced for ERAP1 and ERAP2N, because both enzymes generated the epitope faster than they destroyed it, suggesting that they have the inherent ability to accumulate it (13). This effect was more pronounced for ERAP1 and ERAP2N, because both enzymes generated the epitope at least 100-fold faster than they destroyed it. Of the three enzymes, ERAP1 was by far the most efficient in generating the mature epitope, although ERAP2N was also competitive. Comparing the relative rate of ERAP1- with ERAP2N-mediated epitope generation revealed that ERAP2N can account for ~16% of the ERAP1 activity for this peptide, whereas the ERAP2K activity is negligible compared with that of ERAP1 (Fig. 6B). Taken together, these findings suggest that ERAP2N, but not ERAP2K, can supplement ERAP1 in trimming hydrophobic N termini.

The substrate specificity of ERAP1 has been shown to correlate well with Ag processing and presentation in cultured cells, suggesting that in vitro specificity differences are relevant to in vivo Ag processing (19, 49). To extend these findings to ERAP2 and its allelic content, we used a cell-based Ag presentation assay that has been previously established for ERAP1 (8). We modified this assay to report the effects of ERAP2 allele transfection by cotransfecting with a plasmid coding for the ERAP2 enzyme carrying the desired polymorphism and a minigene encoding a miniprotein that after signal sequence cleavage gives rise to the antigenic peptide precursor ASRHHAFSFR (technical limitations of this assay necessitate the existence of an N-terminal alanine residue; Supplemental Fig. 3). Removal of the N-terminal alanine residue from this peptide generates the mature epitope that can bind onto the nascent MHC class I allele HLA-B27 constitutively produced by this cell line. We measured the changes in epitope presentation on transfection of ERAP2N or ERAP2K compared with control transfected cells that perform processing only by endogenous ERAP1/2. ERAP2N was able to enhance SRHHAFSFR epitope generation.
to a greater degree than ERAP2K (Fig. 6C). In vitro digestion of the same peptide by ERAP2 showed that ERAP2N generated the mature epitope ~2-fold faster than ERAP2K, consistent with the enhanced epitope presentation seen in the cell-based assay (Fig. 6D). This finding suggests that ERAP2 allelic variation can affect Ag presentation in cells. Interestingly, the relative effect of ERAP2N is of a magnitude similar to the effects of ERAP1 allelic variation described before using a similar assay, suggesting that ERAP2 allelic variation can be of equal importance regardless of the accessory role of this enzyme (19).

**Transition-state analogs exert distinct inhibitory effects on the two ERAP2 alleles**

Because the difference in catalytic efficiency between ERAP2N and ERAP2K was due to differences in the $k_{cat}$ of the enzyme, suggesting changes in the recognition of the transition state, we hypothesized that such differences may also apply in the recognition of transition-state analogs. Phosphinic pseudopeptide transition-state analogs have been described before as potent aminopeptidase inhibitors (50). We tested the ability of a phosphinic pseudopeptide of the sequence $L$-$\Psi$-[PO$_2$CH$_2$]$-$LAFKARAF (DG001 peptide) carrying a hydrophobic side chain at its N terminus to inhibit each ERAP2 allele (a more thorough characterization of this class of inhibitors will be described elsewhere [D. Georgiadis, E. Zervoudi, and E. Stratikos, manuscript in preparation]). This compound carries a phosphinic pseudopeptide bond between the two amino acids, resembling the tetrahedral intermediate of the cleavage pathway for the first peptide bond (50, 51). The sequence of this peptide was designed based on known specificity determinants for ERAP1 and ERAP2 (14, 17). The enzymatic hydrolysis of R-AMC by each ERAP2 allele was measured as a function of inhibitor concentration in the solution (Fig. 7A). The DG001 peptide was found to inhibit ERAP2 with good affinity but displayed partial inhibition (Fig. 7A). Strikingly, the DG001 peptide had a much higher affinity for ERAP2N ($K_i = 54 \pm 8 \text{ nM}$) than for ERAP2K ($K_i = 524 \pm 107 \text{ nM}$; Fig. 7A). This 10-fold difference in inhibitor affinity is consistent with the lower activity of ERAP2K for substrates with a hydrophobic N terminus and further supports the hypothesis that the difference between the two alleles lies in the recognition of the transition state. In contrast with these results, inhibition of the two ERAP2 alleles by the substrate analog amastatin was nearly identical (Fig. 7B). This distinction between a transition-state analog and a substrate analog further supports the idea that the differences between the two ERAP2 alleles lie in the mechanism of transition-state stabilization and not substrate affinity. Overall, the differential recognition of a transition-state analog pseudopeptide by the two ERAP2 alleles indicates differences in the catalytic mechanism and suggests that ERAP2 allelic variation should be taken into account for any pharmacological approaches that involve modulation of ERAP2 trimming activity.

**Atomic basis for different trimming between the two ERAP2 alleles**

In an effort to understand the atomic basis of the functional differences between the two alleles, we solved the crystal structure of the ERAP2N allele at 3.2 Å and compared it with the recently determined crystal structure of ERAP2K (Fig. 8, Supplemental Table I) (15). Asn$^{102}$ in ERAP2N is highly conserved among homologous aminopeptidases (Fig. 8A). Although the DG001 analog further supports the idea that the differences between the two ERAP2 alleles lie in the mechanism of transition-state stabilization and not substrate affinity. Overall, the differential recognition of a transition-state analog pseudopeptide by the two ERAP2 alleles indicates differences in the catalytic mechanism and suggests that ERAP2 allelic variation should be taken into account for any pharmacological approaches that involve modulation of ERAP2 trimming activity.

**FIGURE 6.** (A) Epitope generation versus epitope destruction: trimming rates of epitope precursor LSRHHAFSFR (gray bars) and mature epitope SRHHAFSFR (white bars) by ERAP1, ERAP2N, and ERAP2K. (B) ERAP2N can trim the precursor LSRHHAFSFR with a rate corresponding to ~16% of that of ERAP1, whereas ERAP2K is much less efficient. Bars represent the ratio of ERAP2/ERAP1 trimming rates for each ERAP2 variant. (C) HeLa cells, stably expressing HLA-B27, as well as TAP1 ER-transporter blocker (ICP47), were transfected with an ERAP2 variant and an ER-targeted miniprotein that after signal sequence cleavage gives rise to an HLAB27-specific peptide precursor with the sequence ASRHHAFSFR. HLAB27 cell-surface translocation was followed by flow cytometry using an MHC-specific Ab. (D) In vitro trimming rate of the ASRHHAFSFR by ERAP2 alleles. MIF, mean intensity of fluorescence.

**FIGURE 7.** Inhibition of ERAP2N and ERAP2K by a transition-state analog (DG001) (A) and a substrate analog (amastatin) (B). The rate of hydrolysis of the fluorogenic substrate R-AMC was followed in the presence of increasing concentrations of inhibitor for each enzyme. Experimental data were fit to a simple binding model accounting for full or partial inhibition (see Materials and Methods). The calculated constants of inhibition ($K_i$) are shown.
AN ERAP2 SNP CHANGES THE SPECIFICITY OF THE ENZYME

Structural differences between ERAP2 and ERAP2K were also evident in the region capping the S1 pocket (Fig. 8D): 1) the NZ-atom of Lys392 forms a strong salt bridge with the carboxylic group of Asp198 (2.8 Å); and 2) Glu177 assumes a different conformation, away from Lys392, interacting strongly via H-bonding (2.6 Å) with the carboxylic group of Asp888 of domain IV. A water molecule is found close to the previous location of the side chain of Glu177, H-bonding both to NZ of Lys392 and Asp198. These observations suggest subtle changes in the stabilization/recognition properties of the S1 pocket that may underlie changes in specificity. Furthermore, the observed interaction between Glu177 and Asp888 observed only in the case of ERAP2K suggests differences in the interactions between domains II and IV. Reorientation of the relative positions of domains II and IV has been previously reported to be crucial for the catalytic mechanism of the highly homologous ERAP1 (18).

Discussion

Two main ERAP2 SNPs have been associated with disease and proposed to be the result of balancing selection possibly through host-pathogen interactions (27, 30). SNP rs2248374 is a “loss-of-function” variant because it leads to RNA instability and loss of ERAP2 expression (30). This variant leads to a clear functional defect, namely, reduced surface MHC class I expression in B cells, but in contrast with ERAP1 SNPs is not associated with the inflammatory disease ankylosing spondylitis (52). We show in this article that the second ERAP2 SNP, rs2549782, which codes for a single amino acid switch near the enzyme’s active site, is not a loss-of-function variant, but rather a “change-in-function” variant that leads to substrate-specific changes in enzymatic activity, essentially allowing the enzyme to alter its specificity profile for peptide substrates. This unique SNP-related functional change along with the association of this SNP with several human diseases, most notably with resistance to HIV infection, supports the hypothesis that it has arisen by host-pathogen balancing selection and is a component of the immune system variability in natural populations.

The functional consequences of the ERAP2 allelic variation described in this article come in sharp contrast with the effects described for coding SNPs in ERAP1, which have been of much lower magnitude (19, 26, 31). It may, at first view, be difficult to understand how such large differences in activity between ERAP2 alleles are so common in the population (the two ERAP2 alleles are almost equally represented in the human population) (27). It is possible that the proposed “accessory” nature of ERAP2-mediated Ag processing can account for this apparent discrepancy. If, indeed, ERAP1-dependent Ag processing is the dominant activity in the cells, large changes in ERAP2 activity may be easier to tolerate without severely compromising the function of adaptive immunity. Interestingly, the magnitude of Ag presentation changes we report in the cell-based Ag presentation assay is similar between ERAP1 SNPs (as reported in Ref. 19) and between the two ERAP2 variants analyzed in this study. The accessory role of ERAP2 may also present the immune system with an opportunity for evolving new specificities that can modify the antigenic peptide repertoire without severely damaging Ag processing. However, because disease-related immune responses can be dominated by a very small number of antigenic epitopes, it is possible that even an accessory aminopeptidase activity, such as ERAP2, can be dominant in some cases. Unfortunately, the lack of ERAP2 in rodents (although ERAP2 has been hypothesized to have been present in a primate-rodent common ancestor) has limited in vivo experimentation efforts regarding the role of ERAP2 in adaptive immunity in humans.
Modifications of enzyme activity or specificity by mutation can be a useful evolutionary tool for altering metabolic or regulatory pathways, and cancer cells have been shown to use this strategy to gain growth advantages (53). In a notable example of such a change in function, histone methyltransferase EZH2 mutants increase histone methylation in human lymphomas (54). Similar change-in-function mutations in components of the adaptive immune response may be the result of positive selection of individuals that present enhanced responses to specific pathogens during human development. HIV resistance of individuals with a Lys392Lys392 ERAP2 phenotype could represent an example of such positive selection. In this context, the continuously evolving human adaptive immunity can enhance its polymorphic responses to extend away from the well-established variability in MHC binding specificity for antigenic peptides into cellular pathways that are responsible for the generation of those peptides.

Although biochemical and structural analysis suggest a specific mechanism of reduced activity of the ERAP2K allele through reduced stabilization of the N terminus of the peptide, it is less clear how these effects lead to altered specificity. One possibility is that the recognition of substrates by the S1 specificity pocket may be different between the two alleles. ERAP2 recognizes in its S1 pocket positively charged side chains (specifically Lys and Arg) by forming strong electrostatic interactions with residue Asp198 (15, 55). Comparison of the local environment of the S1 specificity pocket of the two ERAP2 variants shows changes in atomic interactions that could affect the energetics of substrate catalysis: 1) the NZ-atom of the bound Lys a in ERAP2K is coming to a closer proximity to Asp198 compared with ERAP2B by 0.7 Å; 2) Glu177 assumes a different conformation, away from Lys a, interacting strongly via H-bonding (2.5 Å) with the carboxylic group of Asp198, leading to a novel interaction with domain IV; and 3) a water molecule is found, close to the previous location of the side chain of Glu177, to interact with Asp198 and Lys a via H-bonds (2.8 and 3.0 Å, respectively). Although the importance of Glu177...Asp198 interaction is difficult to evaluate in terms of substrate recognition or catalytic efficiency of the enzyme, it should be noted that structural rearrangements between domains II and IV have been demonstrated to be important for catalysis in the homologous ERAP1 (18). An alternative or complementary explanation may lie in the suboptimal recognition of hydrophobic side chains by S1 pocket or ERAP2 that leads to unfavorable interactions (14). It is possible that these unfavorable interactions can lead to altered transition-state stabilization in the case of ERAP2K (in which the stabilization of the N terminus is already reduced), making the enzyme more sensitive to optimal S1 pocket occupation and, as a result, more specific. Interestingly, ERAP2K is not the only M1 aminopeptidase with a lysine at position 392; the homologous enzyme aminopeptidase N also has a lysine residue at that location, suggesting that this may be a more general strategy for controlling the stringency of S1 specificity in this family of aminopeptidases (50). The observation, however, that the changes in specificity appear only for the larger, physiologically relevant peptides and not for small, fluorogenic substrates suggests differences in the mechanism of recognition between the two types of substrates. A similar phenomenon has also been described for the highly homologous ERAP1 (17, 18).

In summary, we demonstrate that a common coding polymorphism in the Ag processing aminopeptidase ERAP2 significantly alters its trimming function and provides atomic-level insights on the mechanism behind this effect. The nature and magnitude of the observed functional changes in combination with the wide genetic distribution of this variation suggest that this polymorphic variation constitutes an integral part of the variability of Ag processing in individuals, a variability that can complement the well-established variability in Ag binding and presentation by MHC class I alleles. We furthermore propose that ERAP2K and ERAP2B are treated as distinct aminopeptidase activities in Ag processing when evaluating epitope generation in humans. The importance of polymorphic variation in gene products that regulate the Ag processing and presentation pathway is only now emerging as a key component of the natural variability of the adaptive immune response. A systematic analysis of the functional consequences of these naturally occurring polymorphisms in key components of this pathway can be a valuable tool in both establishing diagnostic tools for disease predisposition and for developing individualized immunotherapies.

Disclosures
The authors have no financial conflicts of interest.

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