The Peptide-binding Motif for the Human Transporter Associated with Antigen Processing

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Summary

Presentation of antigenic peptides by human leukocyte antigen class I molecules is dependent on peptide transport into the endoplasmic reticulum by the transporters associated with antigen processing (TAP) (Germain, R. N. 1994. *Cell.* 76:287–299). This translocation step is currently regarded as permissive for all peptides with COOH-terminal residues capable of binding to HLA class I molecules (Momburg, F., J. Roelse, J. C. Howard, G. W. Butcher, G. J. Hämmerling, and J. J. Neefjes. 1994. *Nature (Lond.).* 367:648–651). In this report, we show that the human transporter selects peptides according to a binding motif based on the strong effects on peptide affinity of the three NH_2-terminal positions and the COOH-terminal residues. TAP favors strongly hydrophobic residues in position 3 (P3) and hydrophobic or charged residues in P2, whereas aromatic or acidic residues in P1, as well as Pro in P1 and P2, have strong deleterious effects. Selection of naturally presented peptides by the transporter is suggested by their higher average affinity for TAP, as compared to nonselected peptides. The TAP preferences in the three NH_2-terminal positions correspond to those of the vast majority of human leukocyte antigen class I alleles, but they represent an obstacle for peptide supply to some alleles, e.g., the B7-like group. We propose that peptides binding to these alleles, and in general, peptides with TAP affinities below a certain threshold, may be transported as extended precursors.

MHC class I proteins present short breakdown products of predominantly cytosolic proteins on the cell surface to cytotoxic T lymphocytes (reviewed in reference 1). Most of these peptides are supplied to newly assembling MHC class I proteins by the heteromeric transporter associated with antigen processing (TAP)1/TAP2[1] transporter complex residing in the membrane of the endoplasmic reticulum (ER). Peptide transport by TAP can be studied using iodinated peptide substrates whose accumulation in the ER is followed grace to an integrated acceptor sequence for ER-specific glycosylation (2–4). As an alternative strategy for the study of TAP function, we recently overexpressed the human TAP complex in the insect cell/baculovirus system. In this system, peptide transport by TAP, as well as direct binding of peptide substrate to the transporter complex, can be measured (5, 6).

Although the functional consequences of a polymorphism in the rat *TAP2* gene clearly demonstrate that TAP selection can modify the spectrum of peptides presented by MHC class I molecules (7), it is not clear whether the human or murine transporters contribute to selection of class I–presented epitopes. Apart from a preference for peptides with a length of 8–16 residues (5, 8, 9), the only selectivity of the transporters known so far concerns the COOH-terminal residue. In this position, the human TAP complex and one rat allele select hydrophobic and positively charged amino acids, while the other rat allele and the murine transporter favor hydrophobic residues only (10, 11). Since the COOH-terminal selectivities of murine and human TAP favor all residues that occur in MHC class I–binding peptides of the respective species, peptide transport by these transporters has been considered to be essentially permissive for all potential MHC class binding peptides.

Large variations in apparent TAP affinities of peptides with identical COOH-terminal residues (5, 12, 13) have been described, however, suggesting that other peptide po-

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; DTT, dithiothreitol; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing.

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sitions may be important for substrate affinity. Apart from a possible deleterious effect of Pro in P1 and P3 (10, 13), previous studies could not identify the variables in the peptide sequence that determined distinct peptide affinities for TAP.

We have used the microsomal peptide binding assay to evaluate the binding affinity of >250 peptides for the human TAP complex. We have defined a binding motif for the human transporter and have shown selection according to this motif among naturally presented peptides. We have also shown how the conflict between the TAP motif and binding motifs for certain HLA class I alleles may be resolved by TAP transport of extended precursors of peptides binding to these HLA class I alleles.

Materials and Methods

Peptides. The peptides used in this study were from various sources. 40 (9-mer) peptides with sequences derived from the hepatitis B virus DNA polymerase and HLA-A2 anchor residues in P2 and P9, five other peptides (11 or 15 mers) derived from various other viral protein sequences, and all poly-Ala peptides (except the poly-Ala variants of the HLA-B27–binding motif) were either purchased as crude material from Chiron Mimotopes (Chiron Corp., Victoria, Australia), or they were synthesized and purified to >95% purity at Cytel as previously described (14). Stocks of these peptides were dissolved at 5 or 10 mM in 100% DMSO. 25 (9 or 10 mer) peptides containing HLA-A2 anchor residues in sequences derived from the human 65-kD glutamic acid decarboxylase protein were obtained from P. Panina-Bordignon (Roche Milano Ricerche, Milan, Italy); synthesis and purification of these peptides are described elsewhere (15). These peptides were dissolved at 2 mM in H_2O, in some cases containing 0.01 N NaOH. Most B27-binding peptides (those used in the QSAR analysis, six peptides with p53-derived sequences, the HSP89a-derived peptide, and the poly-Ala variants of the B27 motif in Table 2) have been described in previous studies (16–18). The 31 peptide variants shown in Table 1 (>90% pure and dissolved at 2 mM in H_2O) were obtained from M. Androlewicz (Yale University, New Haven, CT) (9). The HIV-gag and HIV–reverse transcriptase–derived peptides were obtained from T. Tsomides (Massachusetts Institute of Technology, Cambridge, MA) (19). The HLA-B44 binding peptides are described in reference 20. Peptides that are not specified above were purchased from Genosys (Cambridge, UK), and they were >70% pure and controlled for correct sequence by mass spectrometry analysis; these were all dissolved at 10 mM in 100% DMSO.

HLA Class I α Chain Refolding Assay. An HLA-B27 α chain refolding assay was used to determine the affinity of peptides harboring B27 anchor residues for HLA-B27 (16–18). Briefly, HLA-B27 molecules were purified from lymphoblastoid cell lines and dissociated into α chains and β_2-microglobulin (β_2m) by alkaline treatment and gel filtration. Unfolded free heavy chains were mixed with excess β_2m and test peptides, and the amount of assembled peptide/α chain/β_2m having formed after a 16-h incubation was detected in a radioimmunoassay as inhibition of the binding of ^{125}I-labeled HLA-A2 α/β dimers to an antiserum with specificity for HLA class I α/β dimers.

Soluble MHC–Peptide Binding Assay. The affinity of the HBV polymerase–derived peptides for HLA-A2 and the affinities of poly-Ala peptides containing anchor residues for binding to HLA-A1, A2.1, A3/11, and A24 for their respective HLA class I allele were measured in assays based on the inhibition of binding of a radiolabeled probe peptide to detergent solubilized HLA molecules, as previously described (14, 21, 22). Briefly, HLA molecules were isolated from lysates of lymphoblastoid cells by incubation with solid-phase–coupled HLA class I isotype–specific mAbs. Purified HLA class I α chains were incubated for 2 d at room temperature with excess β_2m, 1–10 nM iodinated probe peptide, and various concentrations of competitor test peptide. At the end of the incubation period, the percent of HLA-bound radioactivity was determined by size exclusion chromatography. As a measure of affinity, the molar concentration of test peptide required for 50% inhibition of binding of the labeled peptide was calculated based on at least two independent assays.

TAP–Peptide Binding Assay. The affinity of peptides for the TAP complex substrate binding site was measured essentially as described previously (5). Microsomes were prepared from Sf9 insect cells harvested 60–80 h after coinfection by recombinant baculoviruses expressing the human TAP1.A and TAP2.A alleles. Sucrose gradient-separated microsomes were washed once in PBS/1 mM dithiothreitol (DTT), resuspended in 1 ml of PBS/DTT per 6–8 × 10^7 cells (starting material), and snap frozen in liquid nitrogen in 200-μl aliquots. Binding assays were performed using PBS/DTT supplemented with 2 mM $MgCl_2$ and 0.1% dialyzed BSA. Serial dilutions of competitor peptide and 15 μl microsome solution were added to 180 μl assay buffer at 4°C in a microfuge tube. Competitor peptides were generally tested in dilutions ranging from 0.1- to 1,000- or from 0.3- to 3,000-fold molar excess relative to radioactive reporter peptide, with stepwise increases by a factor of 10. As a reporter peptide, we used throughout this study R-9-L (RRYNASTEL), whose dissociation constant for the TAP binding site is 4.1×10^{-7} M. Unless otherwise specified, 5 μl peptide R-9-L (labeled with ^{125}I by the chloramin T method to a specific activity of ~7.5×10^7 cpm/nM) was added, resulting in a molar concentration of ^{125}I-R-9-L of ~100 nM. Immediately after the addition of reporter peptide, the vesicles were pelleted by centrifugation at 15,000 *g* for 5 min at 4°C, washed once in 600 μl assay buffer, and finally counted in 100 μl fresh buffer.

Every assay included a duplicate sample without competitor. The cpm corresponding to 50% inhibition of specific binding (I_{50}) were calculated as follows:

$$I_{50} = 0.95*(S_1 + S_2)/2 - 0.95*(S_1 + S_2)/4,$$

with S_1 and S_2 being the cpm of the samples without competitor. The factor 0.95 was introduced to account for the experimentally determined 5% rate of unspecific binding of iodinated R-9-L to control microsomes. The molar concentration of the peptides at which 50% specific inhibition was obtained (IC_{50}) was determined graphically by plotting the cpm in the presence of increasing amounts of competitor on semilogarithmic paper. Under the conditions specified above, the average IC_{50} of autologous competitor peptide R-9-L was 400 nm. To minimize the influence of variations between assays performed on different days and with different batches of reporter peptide or microsomes, affinities are generally indicated as relative values, i.e., multiples of the molar excess of unlabeled autologous competitor R-9-L, which was measured as reference value in all assays.

Chemometric Analysis. The methods and the choice of substituted peptides in the QSAR (quantitiative structure activity relationship) analysis of P3, P5, P6, and P7 have been described previously (17). The PLS (partial least squares) evaluation of the relative TAP affinities of the peptides shown in Table 4 was performed using the program GOLPE (23).

Results

Binding Affinity in Insect Cell Microsomes Parallels Transport Affinity in Human Cells. Using insect cell microsomes overexpressing human TAP1/2 complexes, we have previously shown that selected competitor peptides display equivalent relative affinities in peptide binding and transport assays (6). To further validate the binding assay, we chose to test a series of peptides comprising variants of three peptides known to be naturally presented by HLA-B27 or A3. The affinity of these peptides for TAP had previously been evaluated independently in an assay measuring the accumulation of glycosylated reporter peptide in the ER of streptolysin-O–permeabilized human cells expressing normal levels of the transporter (9). As shown in Table 1, the competition efficiencies of the peptides using both methods were strikingly similar. The rank order of the af-

Table 1. *Comparison of Peptide Transport in Permeabilized Human Cells and Peptide Binding to Insect Cell Microsomes*

No.	Peptide	Length	Sequence	Transport IC_{50}	Binding IC_{50}
				μM	μM
1	Nef 7B, natural	10	QVPLRPMTYK	1.2	11
2	–variant 1	10	QAPLRPMTYK	1.3	47
3	–variant 2	10	QVPLRPMTYA	0.4	14
4	–variant 3	9	QVPLRPMTY	0.6	1.6
5	–variant 4	9	VPLRPMTYK	>100.0	>>100.0
6	–variant 5	8	VPLRPMTY	>100.0	>100.0
7	–variant 6	7	VPLRPMT	>100.0	>>100.0
8	–variant 7	10	KYTMPRLPVQ	18	>100.0
9	–variant 8	11	AQVPLRPMTYKA	0.2	0.2
10	–variant 9	10	AVPLRPMTYA	0.5	5.0
11	–variant 10	10	AVAAAAAAAK	2.1	9.4
12	B27 #1, natural	9	GRIDKPILK	2.5	2.7
13	–variant 1	9	GAIDKPILK	2.2	8.2
14	–variant 2	9	GRIDKPILA	1.4	5.4
15	–variant 3	8	RIDKPILK	8.5	>100.0
16	–variant 4	8	GRIDKPIL	4.4	>100.0
17	–variant 5	7	RIDKPIL	>100.0	>>100.0
18	–variant 6	9	KLIPKDIRG	18	>>100.0
19	–variant 7	11	AGRIDKPILKA	5.1	19
20	–variant 8	9	ARIDKPILA	0.7	0.5
21	–variant 9	9	ARAAAAAAK	3.0	7.0
22	B27 #3, natural	9	RRYQKSTEL	0.5	0.1
23	–variant 1	9	RAYQKSTEL	0.5	0.4
24	–variant 2	9	RRYQKSTEA	0.3	0.7
25	–variant 3	8	RYQKSTEL	>100.0	>>100.0
26	–variant 4	8	RRYQKSTE	2.5	11
27	–variant 5	7	RYQKSTE	>100.0	>>100.0
28	–variant 6	9	LETSKQYRR	1.5	2.3
29	–variant 7	11	ARRYQKSTELA	1.5	2.6
30	–variant 8	9	ARYQKSTEA	0.4	0.2
31	–variant 9	9	ARAAAAAAL	1.2	0.9

The peptides shown in the left column were used as competitors in two assays. First, they were used to compete against ATP-dependent accumulation of labeled reporter peptide ^{125}I-RRYQNSTEL (used at 50 nM in the assay) in the ER of streptolysin O-permeabilized human B cells (Swei). The results of this assay (*center column*) are taken from reference 9, where the procedure is detailed. In this study, the same peptide batches were used to compete for binding of ^{125}I-R-9-L (RRYNASTEL, used at 100 nM) to TAP1/2 insect cell microsomes. The right hand column lists the molar concentration at which the peptides inhibited specific binding of R-9-L by 50%. >100 μM, specific inhibition of <50%, but >20% at 100 μM competitor concentration; >>100 μM, specific inhibition of <20% at 100 μM competitor concentration.

finities of the variants, the range of peptide concentration required for 50% inhibition and the ratio of the IC_{50} of "good" and "bad" competitors were largely equivalent. Shortening of the starting peptide to 7 or 8 mers, as well as reversal of the sequence (with the exception of the reversed variant of B27#3, variant 6), led to drastic reductions in affinity.

Some minor differences, however, should be noted. Replacement of the NH_2-terminal residue (Gln, Gly, Arg; compare variant 8 or 9 to variant 2) by Ala consistently resulted in increased affinity, and replacement of the second residue (Val, Arg; natural vs. variant 1) by Ala resulted in decreased affinity in the binding assay, whereas these changes induced no or a less pronounced effect in the transport system. Moreover, an exchange of Lys or Leu at the COOH terminus by Ala (natural vs. variant 2) slightly increased transport competition, but had the opposite effect on binding to TAP. While the former discrepancies may be interpreted as evidence for a somewhat higher sensitivity of the binding assay, the latter may be caused by a distinct interaction of a COOH-terminal Ala with TAP-unrelated factors (e.g., substrate degradation or active depletion from the ER) that may interfere with peptide accumulation in the ER. Cytosolic or ER-resident factors may also be responsible for the higher apparent transport affinities of peptide nef7B and most of its variants; this peptide may act as an inhibitor of degradation of the reporter peptide, or it may diminish the active depletion of it from the ER. Thus, the peptides generally displayed equivalent competition efficiencies in the two systems, suggesting identical substrate specificities of TAP complexes expressed at physiological levels in the human ER or overexpressed in insect cell microsomes.

Analysis of Single Amino Acid Substitutions in Poly-Ala Peptides. To study the structural requirements for binding of peptide ligands to the TAP substrate binding site, we first tested variants of an HLA-A2 binding 9-mer poly-Ala peptide (ALAKAAAAV). Some of the peptides tested in this and other sets of experiments had purities of <95% (70–95%). We cannot rule out that observed differences may in some cases be caused by contaminating sequences. Conclusions drawn in this study are therefore generally based on observations made with multiple independent sets of substituted peptides.

As shown in Fig. 1, single amino acid exchanges in the starting sequence gave rise to variants with relative affinities for TAP differing by up to 4 logs (compare variant PLAKAAAAV to ALAKAAAAY). As expected, substitutions in the COOH-terminal position had the greatest relative effect (3 logs). Aromatic hydrophobic were followed by aliphatic hydrophobic and then charged residues, while a Gly led to a loss in affinity. Even closely related amino acids such as Tyr and Phe or Leu and Val were associated with distinct binding capacity. Aromatic hydrophobic residues at the COOH terminus confered higher affinity than aliphatic ones; for example, a Tyr at the COOH terminus confered almost 100-fold higher affinity than a Val. Surprisingly, substitutions in the majority of the other positions also could lead to marked alterations in peptide affinity. Strong deleterious effects were observed for Pro in P1 and P2, and Asp and (less pronounced) Phe in P1. In contrast, introduction of aromatic or aliphatic hydrophobic amino acids in several positions, namely (in order of decreasing gain of affinity) in 3, 7, 5, and 6 increased peptide binding capacity. Only in P4 and P8 did minor changes in affinity result from any chosen substitution.

To complement the analysis of the HLA-A2–binding model peptide, we tested variants of peptides containing several other HLA class I–binding motifs (Table 2). At the COOH terminus, the distinct relative affinities of closely related amino acids were confirmed, and additional deleterious effects of Thr and Glu were revealed. Again, aromatic and, in a less pronounced manner, aliphatic hydrophobic residues were prefered to hydrophilic amino acids such as Lys or Ser in P3. Aliphatic hydrophobic residues (especially Val and Ile) and Arg were favored in P2, in which Pro and also Gly demonstrated deleterious effects. Finally, the detrimental effect of an acidic residue in P1 on peptide affinity was confirmed in two more peptide sequences. In summary, substitutions in seven out of the nine positions in the test peptides resulted in gains or losses in peptide affinity, in some cases drastic. Substitutions in the three NH_2-terminal positions attained effects of a magnitude that was similar to that of COOH-terminal substitutions.

Statistical Evaluation of Amino Acid Prevalence in High and Low Affinity Peptides. To obtain additional evidence of the structural ligand requirements for high affinity binding to TAP, we compared the frequency of residues belonging to chemically defined amino acid groups (Table 3 *A*) found in the equivalent position of peptides with high or low affinity for TAP. This analysis was based on the assumption that residues confering high binding capacity would be overrepresented in the group of peptides with high affinity, while residues with detrimental effects on binding would be more prevalent in the group with low affinity. The evaluation was performed only on peptides whose sequences were entirely derived from natural protein sequences, thereby excluding poly-Ala and modified peptides. The group of high affinity binders comprised 44 peptides with a relative affinity of <30; 35 of these were 9 mers. The group of low affinity binders included 44 peptides with a relative affinity of >100 or undetectable binding capacity; 38 of these were 9 mers.

To compare the two groups, we first separately calculated for each position and in each of the two groups of peptides the percentage of amino acids belonging to one of the chemical groups shown in Table 3 *A* relative to the total number of peptides in the respective group of high or low affinity binders. The percentage found in the high affinity group was then divided by the percentage observed in the low affinity group. Thus, a value >1 in Table 3 *A* indicates a preferential occurrence of an individual amino acid or a chemically defined group of residues among the peptides with higher affinity for TAP, and a value <1 indicates a more frequent occurrence among peptides with low affinity. P2 and P9 were not considered in this analysis,

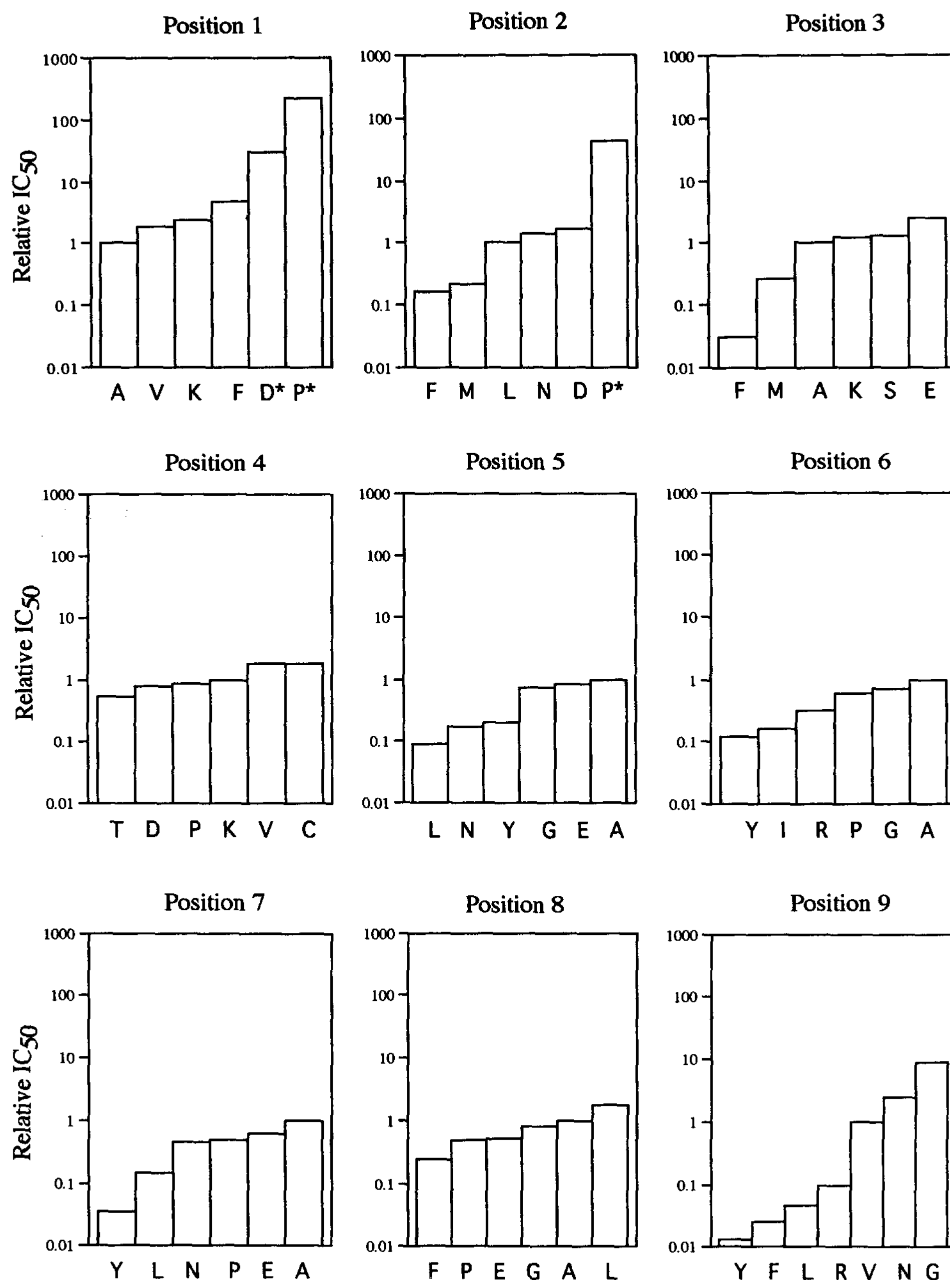

Figure 1. Effect of substitutions in peptide ALAKAAAAV. The nine panels show the effect on TAP affinity of substitutions by the residue indicated below the panel; the position is indicated above it. Binding affinity for TAP is expressed on the logarithmic *y* axis in multiples of the IC_{50} value obtained for the consensus sequence, whose relative affinity was set at 1. Labeled R-9-L reporter peptide was used at 20 nM in all assays, and competitor peptides were used at 0.3–3,000-fold molar excess (7 nM–70 μM). The consensus peptide ALAKAAAAV had an average IC_{50} of 10.8 μM. The relative IC_{50} of variants marked by an asterisk was determined by graphical extrapolation, since 3,000-fold molar excess of these peptides gave <50% inhibition of binding of ^{125}I-R-9-L; these values are therefore rough approximations. All variants of individual positions were tested in the same assay.

since the majority of the peptides had been selected for the presence of canonical HLA-A2 anchor residues in these positions. In peptides with a length of more than nine residues, only P1, P3, and P4 were included in the statistical evaluation. Ratios ≥4 or <0.25 were considered significant and are underlined in Table 3 *A*. Significant favorable and detrimental effects on affinity, as concluded from these ratios, are summarized in Table 3 *B*.

Strongly detrimental as well as favorable effects were observed. The single most significant effect (ratio = 7.1) was the increased binding capacity of peptides carrying an aromatic hydrophobic residue in the third position. Other favorable effects included Pro or an acidic residue in P6, and an aromatic hydrophobic amino acid in P7. Significant unfavorable effects were found more frequently than favorable ones. They included aromatic and acidic residues in P1, Pro in P1 and P3, Gly in P5 and P6, acidic residues in P7, and aliphatic hydrophobic residues in P5. Most of the findings paralleled the observations made with the poly-Ala peptides. Specifically, the strong effects of some residues in P1 and P3 were confirmed, and P4 and P8 were again shown to possess little effect on peptide affinity for TAP.

Chemometric Analysis of the Role of Secondary HLA Class I–Peptide Binding Anchors in TAP–Peptide Interaction. Having observed favorable effects resulting from substitutions in multiple peptide positions, we wondered whether the relative importance of these substitutions could be determined. To reduce the complexity of this analysis, we focused on positions that are known to function as secondary or auxiliary anchors for binding of peptides to HLA class I

Table 2. *Effect of Additional Substitutions in P1, P2, P3, and P9*

Position	Sequence	Relative IC_{50} for TAP	Position	Sequence	Relative IC_{50} for TAP
1	ARAAAAAAA	10	3	ARFAAAIAKK	0.2
	K	152		Y	0.6
	V	200		W	0.8
	E	Undet.		I	1.0
	AIRLKVFVL	0.9		A	1.7
	E	16		V	2.0
				K	7.9
2	AVAKAAAAY	0.5		S	8.3
	I	0.5			
	L	1.0	9	ARAAAAAAA	10
	Y	1.5		E	1,135*
	AVAAAAAAK	24		AYAKAAAAY	1.0
	I	46		F	2.3
	K	57		L	4.6
	L	70		K	23
	Y	70		ALAAAAAAR	29
	N	109		K	70
	T	120		A	114
	G	500		Q	188
	P	2,174*		T	661*
	ARIAAAIAA	2.1		E	1,875*
	A	26			

Poly-Ala peptides with the indicated substitutions in the COOH-terminal or one of the three NH_2-terminal positions were used as competitors in binding assays with 100 nM ^{125}I-R-9-L. Estimated affinities are expressed as multiples of the molar excess of unlabeled competitor peptide R-9-L that was required for 50% inhibition of specific binding of reporter peptide. The average IC_{50} of R-9-L in these assays was 400 nM (corresponding to a molar excess of 4). Thus, a relative IC_{50} of 10 corresponds to an IC_{50} of ~4 μM. A relative IC_{50} marked by an asterisk was determined by graphical extrapolation. Undet., undetectable, i.e. <20% inhibition of specific binding at highest concentration tested.

molecules. Most HLA class I alleles use P2 and the COOH-terminal position as main anchors, while one or several of a group of other positions, namely P3, P5, P6, and P7 frequently act as auxiliary anchors that increase the affinity of peptide binding to HLA by interacting with "secondary pockets" in the peptide-binding site of the HLA molecule.

To assess the relative importance of substitutions in these positions, we used a chemometric approach to QSAR (24). A QSAR analysis entails two steps: first, a statistical method (25) is used to design a series of peptides (nine peptides in this case) that represent the most informative combinations out of all the potential groupings of a chosen number of amino acid parameters in the selected peptide positions. In this case, we have chosen the parameters of hydrophobicity and side-chain bulk (26), and used four amino acids that represent the four possible combinations of high and low values for these parameters: Lys (+/+), Ser (+/−), Phe (−/+), and Val (−/−). Once the affinity of the selected peptides for TAP (or, in chemometric terms, the activity of the selected structures) is known, a quantitative model for the relationship between peptide sequence and TAP affinity can be developed in a second step. This model can be used to predict the affinities of additional peptides for TAP.

The affinities of the nine selected peptides (peptides 1–9) for HLA-B27 and TAP are shown in Table 4 *A*. All peptides carried anchor residues for HLA-B27 in P2 (Arg) and P9 (Lys), and Ala in P4 and P8, and Ser in P1. Several conclusions can be drawn from the evaluation of the peptide affinities for TAP and B27. First, substitutions in the four concerned positions can alter TAP affinity for peptide by 3 logs (compare peptides 3 and 10). Second, a strongly hydrophobic residue in P3 made the by far greatest contribution to peptide affinity for TAP, followed by a hydrophobic residue in P7 and a small side chain in P6; the relative importance of these factors for TAP affinity is expressed in Table 4 *B* as "loadings". Third, the chemometric model based on the test of peptides 1–9 in binding assays allowed an almost exact prediction of the TAP affinity of peptide 10 (0.31 instead of the experimentally observed 0.34), un-

Table 3. *Relative Frequencies of Residues in Positions P1–P8 of Peptides with High or Low TAP Affinity*

A.

Position	1	3	4	5	6	7	8
Y, F, W	0.20	7.1	0.69	1.7	1.1	4.4	3.3
S, T, C	1.4	1.6	0.92	2.5	1.3	0.69	0.86
L, V, I, M	1.6	0.6	0.71	0.24	0.89	1.1	0.93
A	2.7	0.69	0.34	1.5	0.37	2.8	0.28
P	0	0.25	0.28	2.3	4.4	2.2	1.1
G	0.82	0.51	2	0	0	0.55	0.92
Q, N	1	0.34	2.5	0.56	0.55	1.1	0.37
R, K, H	1.9	2.5	1.7	2	1.1	0.89	0.89
E, D	0.20	0.38	1.2	0.92	4.4	0.16	1.7

B.

	1	3	4	5	6	7	8
Increased affinity		Y F W			D, E P	Y, F, W	
Position	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Decreased affinity	Y F W E D P	P		G L V I M	G	D E	

The ratios shown in A were obtained in the following manner: two groups of peptides were formed based on their classification as good to intermediate binders (relative IC_{50} of 30 or lower, $n = 44$) or as poor binders (relative IC_{50} of 100 or higher, $n = 44$). For each peptide position, residues belonging to groups of amino acids shown in the left-hand were counted separately in the two groups. From these numbers, percentages of peptides carrying a particular residue in a specific position and in one of the two groups were calculated. Finally, the percentages observed in the group of good to intermediate binders were divided by those found in the group of poor binders, resulting in the ratio shown in the table. Ratios considered significant are underlined. The significant ratios shown in *A* are graphically summarized in *B*. P2 and P9 were not analyzed since most peptides possessed HLA-A2 anchors in these positions.

Table 4. *Chemometric Analysis of P3, P5, P6, and P7*

A.

Peptide no.	Sequence	IC_{50} in binding to HLA-B27	Relative IC_{50} for TAP
		μM	
1	SRSAKFVAK	>30	23
2	SRFAKKFAK	0.27	2.2
3	SRKAKSSAK	>30	>330
4	SRFAFVVAK	0.06	1.7
5	SRVAVKSAK	6.3	11
6	SRSAFFKAK	1	60
7	SRFASFKAK	0.24	3.8
8	SRKAVSFAK	2.7	22
9	SRVASVFAK	0.15	5
10	SRWAVVWAK	0.05	0.34

B.

	Hydrophilicity		Side-chain bulk	
Position	B27	TAP	B27	TAP
3	−0.71	−0.80	0.28	0.19
5	−0.16	0.01	−0.01	−0.14
6	−0.17	−0.08	−0.42	−0.32
7	−0.38	−0.48	0.31	0.20

In *A*, the sequences of the peptides chosen for the QSAR analysis and their affinities for B27 and TAP are shown. The relative IC_{50}s for HLA-B27 were determined in an HLA class I α chain refolding assay. Since this assay is based on detection of competition, a high value signifies a low affinity. Affinities for TAP are expressed as multiples of the reference IC_{50} of R-9-L competitor peptide, as described in the legend for Table 2. In *B*, the relative contribution of hydrophilicity and shape of the residues in individual positions to the overall affinity of the peptides for the B27 or TAP-binding site are expressed as "loadings." A negative hydrophilicity value indicates a hydrophobic amino acid, while a negative bulk value corresponds to a small side chain.

derscoring the validity of the binding assay and the chemometric evaluation. Finally, the properties of the residues in positions 3, 5, 6, and 7 required for high affinity binding to TAP and B27 appeared strikingly similar, as demonstrated by parallel affinity values for the individual peptides and equivalent loadings (Table 4, *A* and *B*).

The TAP Substrate–binding Site Favors Binding of Peptides with High Affinities for Certain HLA Class I Alleles. Summarizing the results obtained by the three analytical strategies used up to this point, all positions known to represent primary or secondary anchor positions for peptide binding to HLA class I molecules had been shown to be involved in determining the affinity for the transporter complex. It was therefore reasonable to speculate that the distinct requirements for high affinity peptide binding to specific HLA class I alleles may give rise to significant differences in TAP affinity between peptides bearing particular HLA-binding motifs. To approach this issue, we first tested a number of poly-Ala peptides that contained binding motifs for five different HLA class I alleles (Table 5 *A*). All peptides possessed high or intermediate binding affinities (IC_{50} <500 nM) for their respective HLA allele, as determined in HLA–peptide binding assays (not shown). Primary (and secondary for A1 and B27) anchors for various HLA class I alleles in a poly-Ala backbone confered clearly different binding affinities for TAP. The highest affinites were ob-

Table 5. *TAP Affinities of Peptides with High Affinities for Various HLA Class I Alleles*

A.

HLA motif	Sequence	*n*	Relative IC_{50} for TAP (mean)
A1	A(A,L.T)(D,E)(K.A)AAAAY	8	3.9
A2.01	A(L,M,I)AKAAAA(V,I)	4	31.6
A3/11	A(L,V,I,T,G)AAAAAA(K,R)	6	73.1
A24	A(Y,F)AKAAAA(F,L)	3	2.3
B27.05	AR(I,A,W,F)AAA(I,A)AK	6	1.6

B.

HLA motif	Sequence source	*n*	Relative IC_{50} for TAP (mean)
B27	p53	6	9.1
A2	HBV polymerase	13	104.5

C.

Position	HLA-A2.01	HLA-B27.05	TAP
1	Y/F/W	A/G/K/R	A;R/K/H
2	L/M	R	R/V/I;L,Y
3	Y/F/W	Y/F/L/I	Y/F/W
9	V;I	L/F;Y/I/M/R/K	Y/F;L/R

(*A*) The relative IC_{50}s of the indicated number of poly-Ala peptides bearing anchor residues for the listed HLA alleles were tested. The amino acid substitutions performed in anchor positions are indicated in brackets. Each peptide contained only one of the bracketed residues in the indicated positions, and all peptides were 9 mers. (*B*) The average relative IC_{50}s of two groups of peptides derived from the sequence of p53 or HBV polymerase are shown. In the case of the HBV peptides, the indicated average relative IC_{50} was calculated from the values of 11 peptides, since two of these peptides displayed no measureable affinity for TAP. (*C*) The binding motifs for HLA-A2 and B27 are depicted as described in reference 14 for A2 and in references 17 and 36 for B27; only anchor positions and the two positions with the next highest degree of restriction are shown. The TAP motif for the equivalent positions is compiled from the data in Tables 2 and 3. Residues separated by a slash are associated with equal affinity, while residues before a semicolon confer higher affinity than those after it.

served for those motifs that included aromatic hydrophobic residues at the position of a primary (A1, A24) or secondary (B27) anchor, while anchor residues confering high affinity for A2 or A3/11 were associated with markedly lower affinity for the TAP-binding site.

Since most of the poly-Ala peptides lacked secondary anchors naturally found in peptides binding to HLA class I molecules, we undertook a complementary analysis with peptides derived from natural protein sequences. This analysis was performed on six peptides that had been chosen from the p53 sequence for the presence of primary B27 anchors (18) and 13 peptides that had been selected from the sequence of HBV polymerase for the presence of primary A2 anchors (22). Again, all of these peptides exhibited high or intermediate affinity for their respective HLA allele. As shown in Table 5 *B*, the mean affinities of the two peptide groups for TAP differed by a factor of >10. Moreover, two of the A2 binders, but none of the B27 binders, lacked any detectable capacity for binding to TAP. In conclusion, the analysis of peptides derived from natural sequences confirmed the finding of significant differences between average TAP affinities of peptides bearing particular binding motifs for allelic HLA class I products.

An explanation for this difference in average affinities is provided by a comparison of the binding motifs for HLA-A2, B27, and TAP. Table 5 *C* lists the residues associated with high affinity binding to class I or TAP proteins for the four positions that are most restricted and most important for binding in the three biological interactions. With respect to the TAP motif, the A2 motif contains suboptimal residues in the anchor positions P2 and P9 and deleterious amino acids in the auxiliary anchor P1 (see also Table 3), while the B27 motif is entirely constituted of residues that confer high affinity for TAP.

The TAP Affinities of Naturally Processed and Presented Peptides. Having defined a binding motif for the human pep-

tide transporter, we wondered whether any evidence could be found for selection of peptides according to this motif in the HLA class I–associated pathway of antigen processing. We therefore tested the TAP affinities of 21 peptides that are known to be naturally presented by HLA class I molecules (Table 6). None of these peptides were derived from a signal sequence so that all of them were likely to depend on translocation by TAP for access to the ER and HLA class I molecules; only peptide 20 may be generated by degradation of the CD20 transmembrane protein in the ER and thus be independent of TAP-mediated transport. 11 peptides (numbers 1, 2, 4, 7–9, 12, 13, 17, 18, and 20) corresponded to sequences that had been eluted from five different HLA class I alleles, while the others represented T cell epitopes known to be presented by HLA-A2 (six peptides), B27, B44, A3, or Aw68. As shown in Table 6, and in accordance with a recent study (13), naturally processed peptides displayed a wide range of TAP affinities, including the peptide with the highest affinity (1) and one of the peptides with the lowest affinities (21) among all peptides tested in this study. Confirming the previous section's conclusions, all B27-presented peptides had high to intermediate affinities, while some A2-presented peptides displayed low to unmeasurable affinities.

Next, we compared the percentage of peptides with high, intermediate and low TAP affinities in the group of naturally processed peptides to the equivalent percentages in the group of all peptides whose sequence was entirely derived from a natural protein (Table 7). The most striking difference between the two groups concerned peptides with unmeasurable affinities for TAP, which were found rarely among the naturally processed peptides, but in almost a third of the unselected peptides. In addition, the frequency of peptides with high TAP affinities among naturally presented peptides was more than twice greater than that in the group of unselected peptides. Intermediate to low affinities for TAP were frequently found among naturally processed peptides. Thus, this comparison suggests that selection by TAP in class I–associated antigen processing limits ER access to peptides displaying a minimum affinity located in the low to intermediate range, and results in an overrepresentation of peptides with high TAP affinities among naturally presented peptides.

Even though the frequency of low to very low affinity peptides was reduced among naturally presented sequences, we were surprised by the apparent permissiveness of TAP-dependent processing for any such peptide. We speculated that this phenomenon may be explicable if precursors of

Table 6. *TAP Affinities of Naturally Presented Peptides*

Peptide No.	Sequence	Source	HLA restriction	Relative IC_{50} for TAP	Reference
1	EKFEKE**AEMGKGSFY**	EF-1	B44	0.2	20
2	VALREI**RRYQKSTEL**	Histone H3	B27	0.3	37
3	STLELR**SRYWAIRTR**	Flu NP	B27	0.3	37
4	ALNPQN**TVFDAKRLIGR**	Hsc70	A68	0.6	38
5	TQCGYP**ALMPLYACI**	HBV pol	A2	1.0	39
6	TGSEER**SLYNTVATL**	HIV-gag	A2	2.3	19
7	ELFTEL**AEDKENYKKF**	HSP90	B44	3.9	20
8	GVVAGG**GRIDKPILK**	Ripoprot. L8	B27	4.7	37
9	TEYLEE**RRIKEIVKK**	HSP89a	B27	6.7	37
10	FSAGDNPP**VLFSSDFRI**	MAG	A2	8.4	40
11	GFPVTP**QVPLRPMTYK**	HIV-nef	A3	19	8
12	KTVVKK**DVFRDPALK**	Riboprot. L27	A68	29	38
13	LLPSEL**TLWVDPYEV**	TIS21	A2	42	35
14	LAFLQD**VMNILLQYM**	GAD	A2	44	15
15	LAQNRE**ILKEPVHGV**	HIV-RT	A2	54	19
16	AGKDPK**KTGGPIYKR**	Flu NP	A68	56	38
17	GGKDSA**SPRYIFTML**	Topoisom. 2	B7	103	34
18	SGKTYL(S**YLLPAIVHI**)	P68	A2	151(13)	35
19	QNLLQT**EENLLDFVRF**	EBNA3C	B44	218	20
20	WKR(TCS**RPKSNIVLL**)	CD20	B7	595(130)	34
21	FTSI(TN**FLLSLGIHL**)	HPV pol.	A2	Undet.(45)	39

Peptides are listed in order of decreasing affinity for the TAP-binding site. The sequences known to be presented by HLA class I molecules are shown in bold type, joined to 6 or more residues that immediately precede them in the natural protein sequences. For peptides 18, 20, and 21, the sequence and the relative IC_{50} of the tested "precursor peptides" are shown in brackets.

Table 7. *Peptide Affinity Distribution among Naturally Processed and Unselected Peptides*

Relative IC_{50} for TAP	Unselected peptides	Naturally processed peptides
	% (*n* =)	% (*n* =)
>600/Undet.	30.1 (31)	4.7 (1)
51–600	19.4 (20)	28.6 (6)
5.1–50	35.0 (36)	28.6 (6)
0.1–5.0	15.5 (16)	38.1 (8)
Total	100 (102)	100 (21)

The percentages of peptides with the indicated range of relative IC_{50}s in the group of peptides that are known to be naturally processed and presented were calculated and compared to the percentages in the group containing peptides, whose sequence was entirely derived from a natural protein sequence.

such peptides with higher affinities for TAP are translocated into the ER. Intraluminal processing may then generate the peptides that are known to be presented by HLA class I proteins. This hypothesis was supported by the analysis of the TAP affinities of some potential precursor peptides: NH_2-terminal extension of three peptides with very low or undetectable TAP affinities by one or two residues yielded precursors with significantly increased TAP affinity in all of the tested cases (peptides 18, 20, and 21). In summary, the rare frequency of low affinity peptides among naturally processed peptides in conjunction with indirect evidence for a potential transport of precursors of low affinity peptides was compatible with the concept that transport by TAP contributes to peptide selection in class I–associated antigen processing.

Discussion

We have defined a binding motif for the human peptide transporter. To display high binding capacity, 9-mer peptides need to possess (preferably aromatic) hydrophobic or charged residues in both the carboxyterminal and the second position; moreover, a strongly hydrophobic residue in P3 and P7 contributes significantly to binding affinity. Strong detrimental effects on affinity are exerted by Pro in any of the first three positions, by acidic residues in P1, P3, or P7, and by an aromatic hydrophobic amino acid in P1. Although this study is likely to have revealed the most significant features characterizing peptides with high or low affinity for TAP, several questions remain to be addressed. First, we did not test all chemical groups of amino acids in every position. Second, amino acids belonging to the same chemically defined group may have different effects, warranting a systematic study of all possible substitutions in all positions. Third, it will be interesting to study the relative importance of nonterminal positions in longer peptides. Finally, the possibility of mutual interactions between residues in different positions exists.

Although, in analogy to MHC class I molecules, a binding motif for the transporter can be defined, peptide binding to TAP follows rules that appear to be different from peptide binding to MHC class I molecules (27). Most importantly, the TAP motif includes positions with a hierarchy of importance, but no "anchor" positions. In all positions (including the COOH terminus), the array of residues associated with high or intermediate affinity is larger than in any class I anchor position. Moreover, deleterious effects of very unfavorable residues in the relatively most important positions can be compensated by favorable residues in other positions. By contrast, such deleterious substitutions in the anchor positions of HLA class I motifs lead to complete loss of peptide binding to MHC class I. Thus, TAP seems to act as a receptor that selects ligands based on the sum of favorable and unfavorable interactions.

In a hierarchy of importance of favorable influences on binding affinity, the COOH terminus appears to be closely followed by P3 and then P1, P2, and P7. Thus, the positions that appear to contribute most to the binding of peptide to TAP are located at both ends of the peptide. It is tempting to speculate that longer peptides are bound by the TAP substrate–binding site by interaction with their termini, while their central portion bulges out from the binding site.

Like the COOH-terminal preference of human TAP, the preferences for certain residues in the three NH_2-terminal positions correspond well to those of HLA class I alleles, suggesting that the majority of HLA class I–binding peptides are transported as mature peptides that do not need further processing. Among the 32 HLA-A, -B, or -C alleles with known peptide-binding motifs listed in a recent compilation (28), 25 use aromatic hydrophobic residues as anchors (nine alleles) or preferred residues (16 alleles) in position 3. 13 of 21 alleles with known P1 preferences select Ala, Arg, or Lys, which are also favored by TAP. Residues prefered by TAP in P2 represent anchors for 14 and favored residues for three alleles.

However, the preferences of certain HLA class II alleles for peptide ligands, namely of the B7-like group for Pro in 2 and of A2 for aromates in 1, conflict with those of TAP. What are the possible consequences of this finding in a cell expressing various class I molecules? There is no evidence that the rate of surface expression of some HLA alleles is diminished because of inefficient TAP-dependent peptide supply. The TAP motif may, however, influence the degree to which different alleles use a TAP-independent pathway for peptide supply. Both HLA-A2 and -B7 interestingly present a significant proportion of peptides derived from signal peptides (and therefore with TAP-independent access to the ER), as shown by peptide elution and sequencing experiments (29, 30). As an alternative explanation, peptides derived from signal sequences may be more likely to correspond to the A2 and B7 motifs.

Although the hypothesis that an increase in presentation of TAP-independent peptides may compensate for ineffi-

cient HLA allele–specific peptide transport, cannot be ruled out, we favor an alternative concept: intraluminal processing of precursor peptides in the ER. Intraluminal processing of HLA-bound precursor peptides is suggested by several observations. TAP translocates 9–16 mers with equal efficiency (5, 8, 9, 12), HLA class I molecules binding peptides with a length of up to 40 residues can be detected in cell lysates (31, 32), and peptide degradation and processing in the ER have been shown to take place (33). We feel that the finding of naturally presented peptides with very low affinities for the transporter lends additional support to this hypothesis. Moreover, our suggestion of a transport and intraluminal NH_2-terminal processing of extended precursor peptides is compatible with a recent report showing that a T cell epitope can be liberated from a longer precursor by processing in an early secretory compartment by an aminopeptidase activity, but not at all or with much lower efficiency when a carboxypeptidase activity is required (34). Furthermore, peptides that are naturally presented by murine MHC class I molecules and possess very low TAP affinities, but potential precursors with higher TAP affinities have also been found in a recent study by Neisig and coauthors (13). As long as the rules governing the generation of antigenic peptides in the cytosol are not known, however, the suggestion of transport of precursor peptides has to remain speculative.

Will the knowledge of a TAP-binding motif (possibly after further refinements) allow us the more precise prediction of HLA class I–restricted T cell epitopes? A need for further tools in epitope prediction was demonstrated by the finding that high affinity of a peptide for a HLA class I molecule predicts its immunogenicity (as peptide immunogen), but only partly (in <40% of peptides) its antigenicity (i.e., ability to be generated by MHC class I-associated antigen processing and elicit a response in vivo; 22). Clearly, predictions of the antigenic capacity of a peptide based on its TAP affinity are complicated by the fact that the rules governing the generation of transportable and presentable peptides in the cytosol are unknown. If longer precursors of ultimately HLA-binding peptides are regularly generated in the cytosol and then transported by TAP, measurements of TAP affinities of peptides with high affinites for certain HLA alleles may not be sufficient for epitope prediction. Our finding of a relative scarcity of naturally presented peptides with very low affinities suggests that selection by TAP acts at the level of the presented peptides, and that transport of precursors occurs in a minority of cases. Such a bias for the transport of peptides not needing further processing may be imposed by low affinity of precursor peptides for MCI molecules or by a limited efficiency or capacity of the ER-resident processing system.

What degree of selectivity does TAP add to MCI-associated antigen processing? In our selection of peptides with "natural sequences", ~50% had an affinity that failed to reach the presumed threshold. While the percentage of peptides with access to the ER is likely to be increased by posttranslocational processing of precursor peptides, this increase is probably counterbalanced by the loss of other peptides with sufficient affinity for TAP, which cannot be generated in the cytosol and are therefore not available for transport. It is important to bear in mind that the percentage of peptides with low TAP affinities is likely to be higher among entirely unselected peptides, i.e., peptides lacking any anchor residue for binding to HLA class I proteins. Although the HLA-A2–binding motif confers an average TAP affinity that is significantly inferior to the one confered by the B27 motif, the majority of the residues in the A2 anchor positions are associated with moderate to high affinity for TAP. Thus, our panel of "natural peptides" is likely to be biased for high affinity peptides. Even among this selection of peptides, TAP may exclude at least half from the ER.

Why does TAP impose an additional restriction in the selection of potentially immunogenic peptides? First, TAP selectivity may simply represent a byproduct of physical interactions between the transporter and peptides that are required for sufficient affinity and efficiency of the transport process. Alternatively, reduction of the spectrum of peptides that are available for presentation by HLA class I molecules may be useful in limiting the danger of autoimmunity and/or, as recently proposed (35), excessive loss of T cell repertoire in thymic negative selection, both of which may be associated with highly efficient processing. Future studies will show whether the near perfect concordance between the TAP- and the HLA-B27–binding motif is purely coincidental or implicated in the role of B27 as the major HLA class I protein to confer high risk for an autoimmune disease.

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