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HLA-B*2704, an Allotype Associated with Ankylosing Spondylitis, Is Critically Dependent on Transporter Associated with Antigen Processing and Relatively Independent of Tapasin and Immunoproteasome for Maturation, Surface Expression, and T Cell Recognition: Relationship to B*2705 and B*2706[1]

Verónica Montserrat, Begoña Galocha, Miguel Marcilla, Miriam Vázquez, and José A. López de Castro[2]

B*2704 is strongly associated to ankylosing spondylitis in Asian populations. It differs from the main HLA-B27 allotype, B*2705, in three amino acid changes. We analyzed the influence of tapasin, TAP, and immunoproteasome induction on maturation, surface expression, and T cell allorecognition of B*2704 and compared some of these features with B*2705 and B*2706, allotypes not associated to disease. In the tapasin-deficient .220 cell line, this chaperone significantly influenced the extent of folding of B*2704 and B*2705, but not their egress from the endoplasmic reticulum. In contrast, B*2706 showed faster folding and no accumulation in the endoplasmic reticulum in the absence of tapasin. Surface expression of B*2704 was more tapasin dependent than B*2705. However, expression of free H chain decreased in the presence of this chaperone for B*2705 but not B*2704, suggesting that more suboptimal ligands were loaded on B*2705 in the absence of tapasin. Despite its influence on surface expression, tapasin had little effect on allorecognition of B*2704. Both surface expression and T cell recognition of B*2704 were critically dependent on TAP, as established with TAP-deficient and TAP-proficient T2 cells. Both immunoproteasome and surface levels of B*2704 were induced by IFN-γ, but this had little effect on allorecognition. Thus, except for the differential effects of tapasin on surface expression, the tapasin, TAP, and immunoproteasome dependency of B*2704 for maturation, surface expression, and T cell recognition are similar to B*2705, indicating that basic immunological features are shared by the two major HLA-B27 allotypes associated to ankylosing spondylitis in human populations. ***The Journal of Immunology,*** **2006, 177: 7015–7023.**

Human MHC class I molecules constitutively bind and present at the cell surface a highly diverse repertoire of endogenous peptides derived from the degradation of cellular proteins. The peptide processing-loading pathway involves a series of proteins that together determine the stable surface expression and Ag presentation of the class I molecule. The major protease involved in the generation of class I-bound peptide repertoires is the proteasome, a multicatalytic and multisubunit complex whose composition and activity is modulated by IFN-γ. This cytokine induces the expression of various proteasome subunits (β1i, β2i, β5i), which substitute the corresponding constitutive ones (β1, β2, β5), giving rise to a modified complex designated as immunoproteasome (1). Peptides generated by proteasomal degradation can be further subjected to amino peptidase-mediated trimming before reaching the optimal size for HLA class I binding (2–5). Peptide transport into the lumen of the endoplasmic reticulum (ER)[3] is regulated by TAP. This heterodimeric protein is responsible for much of the peptide supply to the nascent HLA class I molecules, and its absence has drastic effects on their surface expression (6), although in an allotype-dependent way (7). The peptides reaching the ER are bound to the class I molecule in a process of assisted loading involving multiple proteins collectively known as the peptide-loading complex. This includes, besides the HLA class I heterodimer and TAP, three additional proteins: calreticulin (8), ERp57 (9–11), and tapasin (Tpn) (8, 12). This latter chaperone bridges the class I molecule to TAP, upregulates TAP levels (13), favors peptide binding to TAP (14), and contributes to editing (15), optimizing (16, 17), and facilitating (18) peptide loading. Its effect on HLA class I-bound peptide repertoires is also allotype dependent (19, 20).

The peptide-loading properties of HLA-B27 have attracted much interest due to the strong association of this molecule with susceptibility to ankylosing spondylitis (AS) and other spondyloarthropathies (21, 22). The pathogenetic role of HLA-B27 has been thought to be related to its peptide-presenting properties (23), but more recently, both its folding features, which may give rise to an unfolded protein response upon accumulation of the H chain in the ER (24–26) and the surface expression and immune recognition of β_2-microglobulin (β_2m)-free forms of the molecule, such as H chain homodimers (27), have been proposed as putative pathogenetic features. So far, most studies dealing with the early events

Centro de Biología Molecular Severo Ochoa (Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid), Facultad de Ciencias, Universidad Autónoma, Madrid, Spain

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[2] Address correspondence and reprint requests to Dr. José A. López de Castro, Centro de Biología Molecular Severo Ochoa, Facultad de Ciencias, Universidad Autónoma, 28049 Madrid, Spain. E-mail address: aldecastro@cbm.uam.es

[3] Abbreviations used in this paper: ER, endoplasmic reticulum; Tpn, tapasin; AS, ankylosing spondylitis; β_2m, β_2-microglobulin; Endo H, endoglycosidase H.

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of HLA-B27 maturation and peptide loading have been conducted with B*2705, the prototype HLA-B27 molecule and the most abundant subtype in Caucasians. These studies have demonstrated a strong dependence of B*2705 on TAP (28), and a relative, but not total, independence of Tpn (15, 16, 19).

B*2704 is the most frequent subtype in Eastern Asian populations and, like B*2705, is strongly associated with AS (29, 30). B*2704 differs from B*2705 by three amino acid changes, S77D, E152V, and G211A. The two former ones are located in the peptide-binding site and affect peptide specificity and T cell recognition (31, 32). Surface expression of B*2704 is lower than other HLA-B27 subtypes in the absence of Tpn, a phenotype that can be reverted by mutations at the polymorphic positions 116 and 152 (33). In this study, we analyzed the influence of TAP, Tpn, and induction of immunoproteasome on maturation, egress from the ER, surface expression and T cell recognition of B*2704, and compared some of these properties with B*2705 and B*2706. The latter subtype is present mainly in populations of Southeast Asia and the Pacific, is not associated to AS (34–36), and differs from B*2704 only by the H114D and D116Y amino acid changes (37, 38).

Materials and Methods

Cell lines and mAb

Hmy2.C1R (C1R) is a human lymphoid cell line with low expression of its endogenous class I MHC Ags (39, 40). T2 is a human mutant cell line with the MHC class II region, including the TAP genes, completely deleted (41). These cells express very low levels of HLA-A2, -B51, and -Cw1 on their surface (42). T2-TAP (a gift from Dr. F. Momburg, German Cancer Research Center, Heidelberg, Germany) is a T2 cell line transfected with the human *TAP1*/*TAP2* genes (43). C1R transfectants expressing HLA-B27 subtypes were previously described (44). T2-B*2704, T2-TAP-B*2704 transfectants, were obtained as follows. The B*2704 genomic DNA cloned in pBR322 was cotransfected with pSV2*neo* (in T2) or Bluescript M3 carrying the puromycin-resistance gene (T2-TAP) by electroporation at 500 μF and 260 V. The human B lymphoblastoid cell line 721.220 (.220) is a gamma-irradiation mutant that lacks both *HLA-A* and *-B* genes and expresses a truncated and nonfunctional tapasin protein (45, 46). The .220 cells transfected with either B*2704 alone (.220-B*2704) or with both B*2704 and human Tpn (.220-Tpn-B*2704) were provided by Drs. J. McCluskey and A. Purcell (University of Melbourne, Parkville, Victoria, Australia). The .220-B*2706 transfectant was a gift from Dr. R. Colbert (Cincinnati Children's Hospital, Cincinnati, OH). All the cell lines were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS.

The mAb used in this study were ME1 (IgG1, specific for HLA-B27, B7, B22) (47) and HC10 (IgG2a, specific for HLA class I H chain not associated to β_2m) (48). Both mAb have high affinity for their respective Ags. Other mAb used for Western blot analyses are described below.

Quantitative PCR of HLA-B27 and Tpn

Quantitative RT-PCR was used to assess the expression levels of Tpn and HLA-B27 in the transfectant cells used in this study. Quantification of Tpn was done as previously described (49). RT-PCR of HLA-B27 was conducted by a similar procedure as follows. Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies) and 1 μg was reverse transcribed using MultiScribe reverse transcriptase and the Archive kit reagents (Applied Biosystems) in a final volume of 100 μl. RT-PCR was performed with 10 ng of each cDNA in 96-well plates using a sequence detection system ABI PRISM 7000 (Applied Biosystems). Samples were analyzed in triplicate with the primers forward and reverse 5′-TCTGTGC CTTGGCCTTGC-3′ and 5′-GGGCGCCGTGGATAGAG-3′, corresponding to nucleotides 271–288 and 215–230 of HLA-B27, respectively, and the HLA-B27-specific TaqMan probe FAM-5′-CGGGAGACACAGATC-3′, corresponding to nucleotides 256–269, as well as with the ribosomal 18S-specific FAM-labeled probe Hs99999901-s1 (Applied Biosystems). PCR amplification was performed at 60°C for 40 cycles using TaqMan universal PCR master mix (Applied Biosystems). Cycle threshold values were calculated using automatic adjustment of the threshold.

Pulse-chase experiments

Cells were incubated with L-Met/L-Cys-free DMEM supplemented with 10% FBS and 2 mM L-glutamine for 45 min at 37°C. Cells were pulse labeled with 500-1000 μCi/ml [^{35}S]methionine-cysteine (Amersham Biosciences) at 37°C for 15 min, and chased with complete RPMI 1640 medium supplemented with 1 mM cold L-Met, L-Cys at 37°C for the indicated times. At each time point, cells were spun down, resuspended in 50 μl of PBS, frozen in liquid nitrogen and stored at −80°C. Cells were lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$) containing a mixture of protease inhibitors (Complete Mini; Roche). Lysates were centrifuged at 14,000 rpm for 10 min at 4°C, precleared three times for 60 min with CL-4B beads (Sigma-Aldrich) and 3 μl of normal mouse serum, and immunoprecipitated with an excess of the ME1 and HC10 mAb and protein A-Sepharose beads (Sigma-Aldrich). Immunoprecipitates were normalized to equal TCA-precipitable ^{35}S-labeled protein, washed three times with Nonidet P-40 washing buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) and analyzed by SDS-PAGE. Endoglycosidase H (Endo H) (New England Biolabs) was added to the immunoprecipitates according to the manufacturer's instructions. Samples were visualized by fluorography and exposed to Agfa-Curis RP2 Plus films. For quantization of the bands, the autoradiograms were scanned and then analyzed using the TINA2.09e software (Isotopenβgeräte).

Flow cytometry

Approximately 3×10^5 cells were washed twice in 200 μl of PBS and resuspended in 50 μl of purified mAb, at saturating conditions (ME1:10 μg/ml; HC10:50 μg/ml). After incubating for 30 min, cells were washed twice in 200 μl of PBS and resuspended in 50 μl of FITC-conjugated anti-mouse IgG rabbit antiserum (Calbiochem-Novabiochem), incubated for 30 min, and washed twice in 200 μl of PBS. All procedures were done at 4°C. Flow cytometry was conducted on a FACSCalibur instrument (BD Biosciences).

In other experiments, .220 cell lines were washed twice with serum-free AIM medium (Invitrogen Life Technologies), seeded to 3×10^5 cells/well in a final volume 100 μl of the same medium alone, or containing peptide (100 μM) and/or human β_2m (1.25 μg/μl). Cells were incubated at 26°C, and subjected to flow cytometry as above.

*HLA-B*2704-specific CTL and cytotoxicity assay*

Alloreactive CTL clones against B*2704 obtained by limiting dilution from two HLA-B27-negative donors: V (HLA-A24, 29; B44, 57; Cw7; DR7, 53) and M (HLA-A1, 2; B7, 18; Cw5,7; DR7, 17, 52, 53) were previously described (32). T cells were grown in IMDM with glutamax I (Invitrogen Life Technologies), supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin sulfate, and 0.05 mg/ml gentamicin (all from Sigma-Aldrich) and 14% FBS (Invitrogen Life Technologies), and were restimulated weekly in the presence of 30 U/ml rIL-2 (a gift from Hoffmann-La Roche, Nutley, NJ). Their cytolytic activity against B*2704 target cells was measured using a standard ^{51}Cr release cytotoxicity assay (50).

IFN-γ treatment

C1R-B*2704 cells were treated with 100 U/ml IFN-γ (Roche) at various times. Aliquots were removed after 0, 3, and 6 days to analyze the surface expression of B*2704 by flow cytometry. Another aliquot was used for cytotoxicity assay. A third aliquot was used to analyze the proteasome subunit composition by Western blot.

Western blot

For detection of Endo H-sensitive and -resistant HLA class I H chains on whole lysates, the following procedure was used: $\sim 1.5 \times 10^6$ cells were lysed in 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, containing a mixture of protease inhibitors (Complete Mini; Roche). For each cell line, 100 μg of protein was divided into two aliquots. One was treated with Endo H (New England Biolabs) and the other was left untreated. After SDS-PAGE, HLA class I H chains were revealed with the HC10 mAb, using the peroxidase-conjugated sheep anti-mouse IgG Ab NA 931 (Amersham Biosciences) as secondary Ab. For detection of inducible proteasome subunits, a similar procedure was used, with the following modifications: $\sim 1.5 \times 10^6$ C1R-B*2704 cells were removed after 0, 3, and 6 days of the addition of IFN-γ. The pellet was lysed by boiling in the loading buffer for SDS-PAGE and distributed in two aliquots, for detection of β1i and β1 plus tubulin. The mAb used were PW 8840, PW 8140 (Affinity), and anti-γ-tubulin (Sigma-Aldrich), respectively, and the same secondary Ab as above.

Results

*The surface expression and dissociation of B*2704, B*2705, and B*2706 are Tpn dependent*

In a previous study (33) using transient expression of HLA-B27 subtypes on Tpn-negative and Tpn-positive .220 cells, the surface expression of the B*2704 heterodimer was more Tpn dependent than B*2705 or B*2706. In this study, we compared the Tpn dependency of B*2704, B*2705, and B*2706 using stable transfectants of these subtypes on Tpn-negative and, except for B*2706, Tpn-positive .220 cells. Tpn expression in the B*2705 transfectant was ~3-fold higher than in the B*2704 counterpart, as determined by quantitative RT-PCR (Fig. 1*A*). Expression of HLA-B27 in the various transfectants was also determined by the same technique (Fig. 1*B*). The surface expression of the B*2704, B*2705, and B*2706 heterodimers and free H chains in .220 transfectants was measured by flow cytometry with the ME1 and HC10 mAb, respectively (Fig. 1*C* and Table I). In the absence of Tpn, the heterodimer:H chain ratio, as measured by the ME1:HC10 fluorescence ratio, was ~1.7, suggesting that a high percentage of the B*2704 expressed on the cell surface was in a β_2m-free form. In the presence of Tpn, the B*2704 heterodimer expression was increased ~2-fold, and the HC10-associated fluorescence was not significantly altered. This resulted in a 2-fold increased ME1:HC10 fluorescence ratio (~4), up to levels comparable to those observed in Tpn-positive C1R-B*2704 transfectant cells (Table I). These results indicate that Tpn makes a significant contribution to the cell surface expression and stability of the B*2704 heterodimer, confirming previous observations (33). B*2705 was less dependent on Tpn than B*2704 for surface expression. However, the HC10-associated fluorescence was decreased by ~2-fold in the presence of the chaperone, indicating that, like B*2704, the B*2705 heterodimer expressed in the absence of Tpn was less stable at the cell surface, as previously reported (49). The ME1:HC10 fluorescence ratio for B*2706 on .220 cells was similar to that of B*2705. A Tpn-positive counterpart was not available for this subtype, which precluded an assessment of the influence of Tpn on the surface expression and stability of B*2706 in .220 cells. However, we have previously reported that the ME1:HC10 fluorescence ratio in the Tpn-positive C1R-B*2706 cells was ~7 (51) (Table I). Although comparisons between different cell lines must be considered with caution, this is compatible with a similar effect of Tpn on surface expression of B*2706 and B*2705.

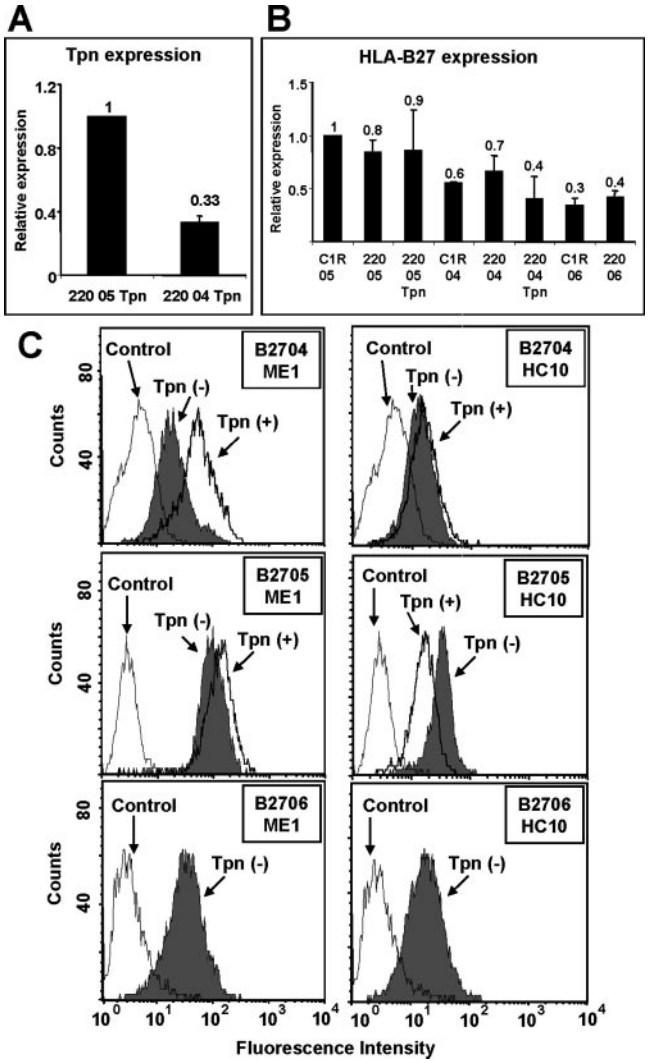

FIGURE 1. Expression of B*2704, B*2705, and B*2706 in .220 transfectant cells. *A*, Relative expression of Tpn in Tpn-positive .220-B*2705 and .220-B*2704 transfectant cells, assessed by quantitative RT-PCR. Data are mean ± SD of three independent experiments. *B*, Expression of B*2705, B*2704, and B*2706 in C1R and .220 transfectant cells, assessed by quantitative RT-PCR. Data are mean ± SD of three independent experiments and are relative to the expression of B*2705 in C1R cells. *C*, Surface expression of the B*2704, B*2705, and B*2706 heterodimers (ME1) and free H chains (HC10) on Tpn-negative (gray) or Tpn-positive (thick lines) .220 transfectant cells. Negative control (thin lines) is the fluorescence obtained with the secondary Ab alone. Data from a representative experiment, of three independent ones, are shown. Mean ± SD of the three experiments, and the corresponding ME1:HC10 and Tpn^+:(−) fluorescence ratios, are shown in Table I.

Table I. *Surface expression of B*2704, B*2705, and B*2706 heterodimers and free class I H chains on .220-transfectant cells*[a]

Cell	ME1	HC10	Me1:HC10 Ratio
.220	6 ± 1	8 ± 2	0.8 ± 0.4
.220-Tpn	16 ± 5	6 ± 2	3.0 ± 0.7
.220-B*2704	48 ± 29	26 ± 18	1.7 ± 0.1
.220-Tpn-B*2704	96 ± 67	24 ± 12	3.8 ± 0.8
Tpn (+):(−) ratio	2.1 ± 0.3	1.0 ± 0.2	
.220-B*2705	82 ± 21	31 ± 6	2.7 ± 0.5
.220-Tpn-B*2705	115 ± 27	16 ± 1	7.3 ± 1.3
Tpn (+):(−) ratio	1.4 ± 0.1	0.5 ± 0.1	
.220-B*2706	57 ± 20	21 ± 4	2.7 ± 0.7
C1R-B*2704			6 ± 3[b]
C1R-B*2705			6 ± 3[b]
C1R-B*2706			7 ± 2[b]

[a] Mean channel fluorescence ± SD of three to four experiments.
[b] These data were previously reported (51) and are shown here only for comparison. Results are mean ± SD from 8 to 12 experiments.

In the presence of a high-affinity natural ligand of B*2704 (RRYQKSTEL), a small increase of ME1-associated fluorescence was observed in the .220, but not in the .220-Tpn, transfectants. The HC10-associated fluorescence was not altered in either cell line (Fig. 2). These results suggest that the β_2m-free B*2704 H chains in these transfectants are largely in irreversible forms. The exogenous addition of β_2m did not have any significant effect on B*2704 expression (data not shown).

*Tpn influences the folding extent of B*2704 and B*2705 in .220 cells*

The kinetics of heterodimer formation and its Tpn dependence was analyzed by pulse-chase labeling of .220-B*2704 and .220-Tpn-B*2704 transfectant cells, in the corresponding B*2705 transfectants and in .220-B*2706 cells. For B*2704 and B*2705, the ratio

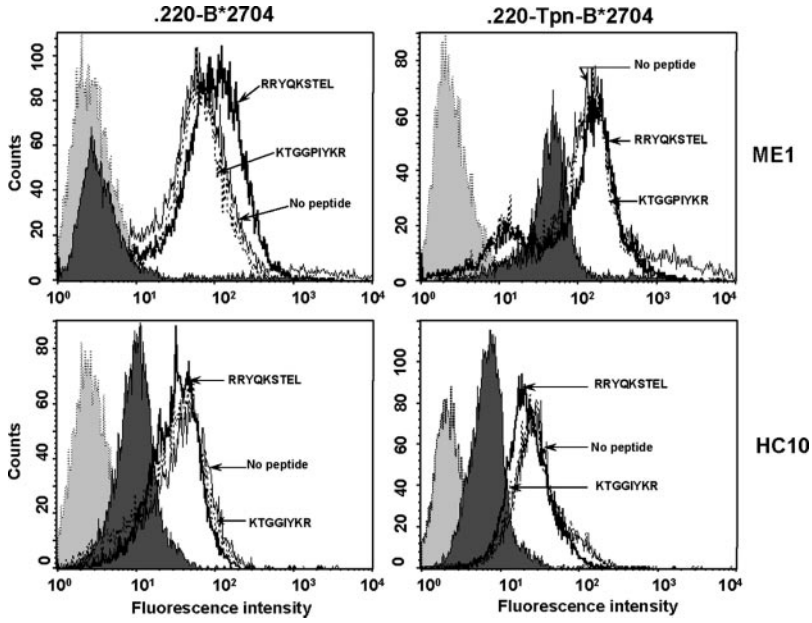

FIGURE 2. Surface expression of B*2704 on .220 cells with and without Tpn. Flow cytometry analysis of .220-B*2704 (*left panels*) and .220-Tpn-B*2704 (*right panels*) with ME1 (*upper panels*) and HC10 (*lower panels*) mAb. The analysis was performed in the absence of added peptide (thin lines), in the presence of the mock peptide KTGGPIYKR (dotted lines) and in the presence of the natural B*2704 ligand RRYQKSTEL (thick lines). The shaded histograms correspond to the controls with only secondary Ab (dotted line and shaded) and to untransfected .220 and .220-Tpn cells (continuous line and shaded). A representative experiment of three independent ones (two for the controls) is shown. Mean channel fluorescence ± SD of the three experiments without, with mock, or with the relevant peptide were the following for ME1 and HC10, respectively: .220-B*2704: 107 ± 27/41 ± 8; 114 ± 32/41 ± 8, and 141 ± 21/40 ± 7; .220-Tpn-B*2704: 206 ± 46/23 ± 14; 211 ± 45/24 ± 14; 211 ± 35/19 ± 9.

of free to β_2m-associated H chain, as established by immunoprecipitation with HC10 and ME1, respectively, was very low in the presence of Tpn and significantly increased in the absence of this chaperone (Fig. 3*A*), suggesting that formation of the B*2704 and B*2705 heterodimers, relative to the total amount of free H chains, was much less efficient in the absence of Tpn, leading to substantial accumulation of free H chains in the ER. However, the maturation kinetics of the B*2704 and B*2705 heterodimers was not significantly affected by Tpn, as judged by the appearance of fully glycosylated H chains, revealed by a slightly lower electrophoretic mobility of the band precipitated with ME1. In contrast, B*2706 did not significantly accumulate in the ER of .220 cells, even in the absence of Tpn (Fig. 3*A*). Analogous experiments conducted on the Tpn-positive C1R-B*2706 cells also showed no accumulation of the B*2706 H chain in the ER (B. Galocha and J. A. López de Castro, unpublished observations; manuscript in preparation), suggesting that Tpn has little influence on the folding efficiency of B*2706. This is consistent with the observation that interaction of B*2706 with the peptide-loading complex is much less efficient than for other HLA-B27 subtypes (33). The relative amount of Endo H-resistant and Endo H-sensitive MHC class I H chains in the steady state was determined in .220-B*2704, B*2705, and B*2706 and, except for this latter subtype, in their Tpn-positive counterparts, by Western blot analysis of whole lysates with HC10. As expected, the ratio of Endo H-resistant to Endo H-sensitive H chains was significantly higher in the presence of Tpn for B*2704 and B*2705.

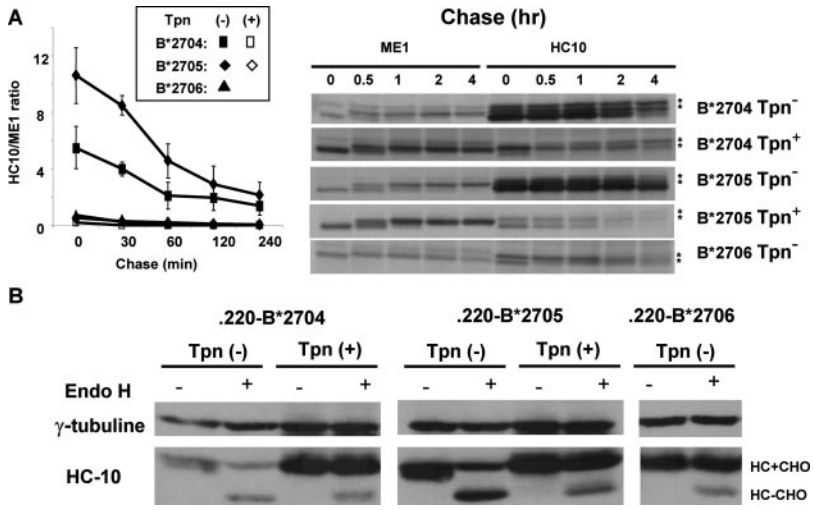

FIGURE 3. *A*, Folding kinetics of B*2704, B*2705, and B*2706 in .220 cells. The cells were pulse-labeled for 15 min with [^{35}S]Met/Cys and then chased for the indicated time intervals. After lysis, the HLA-B27 heterodimer was immunoprecipitated from one-half of the sample with the ME1 mAb. The HLA class I H chain was immunoprecipitated from the other half of the sample with the HC10 mAb. The immunoprecipitates were analyzed by SDS-PAGE, densitometered, and the ratio of H chain immunoprecipitated with HC10 and ME1 (HC10:ME1 ratio) was calculated and plotted vs time. Results are means of three or four experiments for B*2704 and B*2706, and two experiments for B*2705. Representative experiments for each cell line are shown in the *right panel*. ∗, Nonspecific bands. *B*, Western blot analysis of B*2704, B*2705, and B*2706 in whole lysates of .220 cells with the HC10 mAb, with and without Endo H treatment. A representative experiment, of two (for B*2705 and B*2706) or three (for B*2704) independent ones, is shown. The ratio ± SD of the Endo H-resistant (HC+CHO) to Endo H-sensitive (HC−CHO) species were the following: .220-B*2704, 1.1 ± 0.1; 220-Tpn-B*2704, 2.5 ± 0.9; 220-B*2705, 1.0 ± 0.1; 220-Tpn-B*2705, 1.8 ± 0.5; 220-B*2706, 2.1 ± 0.6.

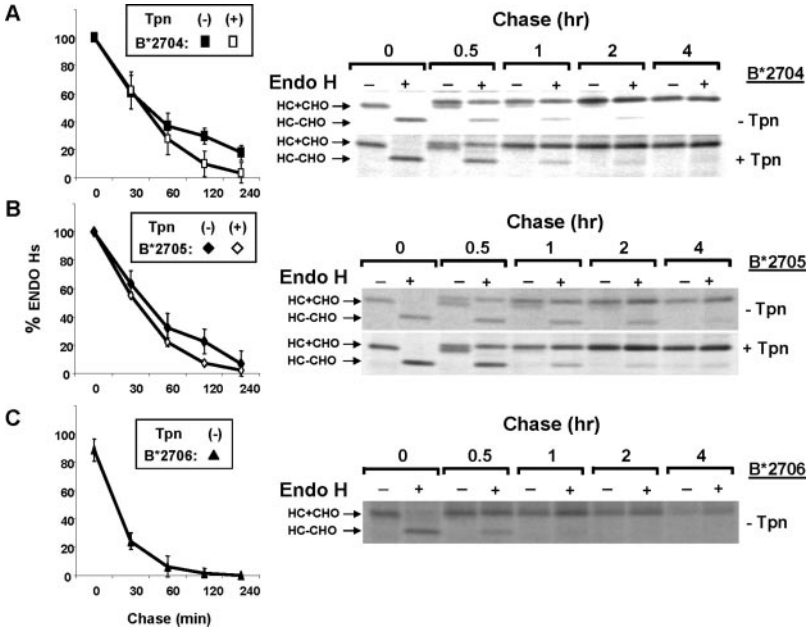

FIGURE 4. The B*2704 (*A*), B*2705 (*B*), and B*2706 (*C*) heterodimers were immunoprecipitated from cell lysates of .220 (filled symbols) or .220-Tpn transfectants (open symbols) at various times with ME1. Immunoprecipitates were divided into two aliquots. One was left untreated and the other was digested with Endo H. The H chains were analyzed by SDS-PAGE, and the Endo H-resistant and -sensitive forms were quantified by densitometry. The percentage of Endo H-sensitive forms relative to the total H chain immunoprecipitated with ME1 was plotted vs time. Results are means of three to six independent experiments. Representative experiments are shown to the right of each plot. The times (in minutes) of acquisition of 50% Endo H resistance were the following: .220-B*2704, 49 ± 4; .220-Tpn-B*2704, 36 ± 7; .220-B*2705, 42 ± 11; .220-Tpn-B*2705, 30 ± 2; .220-B*2706, 13 ± 1.

However, in B*2706, the percentage of Endo H-resistant H chain in the absence of Tpn was similar to that of the other subtypes in the presence of this chaperone (Fig. 3*B*).

*Tpn has little influence on the export rate of B*2704 and B*2705 from the ER*

The influence of Tpn on the export rate of B*2704, B*2705, and B*2706 was estimated in the corresponding Tpn-negative and, except for B*2706, Tpn-positive .220 transfectants by measuring the acquisition of resistance of the B27 H chains to Endo H digestion (Fig. 4). Both B*2704 and B*2705 showed only a slight increase in the export rate in the presence of Tpn, indicating that, in contrast to its effect on the efficiency of folding, this chaperone has little influence on the export rate of the folded heterodimer for these two subtypes, in agreement with their similar maturation kinetics revealed by the glycosylation pattern (Fig. 3*A*). Similar results were reported for B*2705 (52). In contrast, the export rate of B*2706 in the Tpn-negative .220 cells was significantly shorter than for B*2704 and B*2705. The export rate of B*2706 on C1R-B*2706 transfectants (our unpublished observations; B. Galocha and J. A. López de Castro, manuscript in preparation) was very similar to that on .220-B*2706 cells (time of acquisition of 50% Endo H resistance: 14 and 13 min, respectively), suggesting that Tpn does not influence the export rate of this subtype. A faster export rate of B*2706 relative to other subtypes in the presence of Tpn was also observed in another Tpn-positive cell line 721.221 (33).

*T cell allorecognition of B*2704 is Tpn independent*

A total of 25 alloreactive CTL clones directed against B*2704 were tested for recognition of this allotype expressed on both .220 and .220-Tpn cells. The B*2704 specificity of the CTL clones was established on the basis of their lysis of C1R-B*2704 targets, but not of untransfected C1R cells. All the CTL clones tested lysed both the Tpn-negative and the Tpn-positive .220 transfectant targets (Table II). A majority of these CTL lysed both targets with similar efficiency, but a few (i.e.: CTL 66, 116, and 118) showed a somewhat increased lysis of Tpn-positive targets. CTL 118 could be tested at various E:T ratios for lysis of Tpn-deficient and Tpn-proficient targets (Fig. 5), confirming that this CTL clone lysed B*2704 targets more efficiently in the presence of Tpn. Because alloreactive CTL recognize a diverse set of the alloantigen-bound natural ligands (53–55), these results suggest that a large majority of the B*2704-bound peptide repertoire is presented in the absence of Tpn, but some allospecific peptide epitopes may be more efficiently presented in the presence of Tpn. These results are consistent with Tpn-dependent quantitative alterations of the B*2704-bound peptide repertoire, as determined for B*2705 (15). Although 24 of the 25 CTL clones, except V26, were from the same donor (M) the high

Table II. *Specific cytotoxicity of anti-B*2704 CTL, towards .220-B*2704 with and without Tpn*[a]

CTL	C1R	C1R-04	.220-04	.220-04-Tpn
5	4	62	62 (2)	62 (2)
7	7	60	53 (2)	51 (2)
20	4	52	60	40
25	8	74	53 (2)	47 (2)
28	6	68	73	62
42	0	23	31	37
45	0	51	60 (2)	62 (2)
52	0	44	73	67
57	0	58	54 (2)	58 (2)
62	0	60	43 (2)	49 (2)
66	0	100	50	76
68	10	54	49 (2)	46 (2)
71	8 (2)	49 (2)	38 ± 8 (3)	46 ± 18 (3)
72	3	43	50 (2)	54 (2)
84	16	60	67 (2)	68 (2)
85	12	67	69 (2)	74 (2)
89	10	89	42	47
95	9	83	25 (2)	32 (2)
96	1 (2)	66 (2)	57 ± 9 (4)	66 ± 13 (4)
106	20	100	50 (2)	68 (2)
111	8	61	71 (2)	75 (2)
116	4	62	43 ± 1 (3)	60 ± 16 (3)
118	1 (2)	50 (2)	29 ± 9 (4)	42 ± 18 (4)
131	10	68	56 ± 5 (4)	64 ± 9 (4)
V26	0	46	56	56

[a] Data are expressed as the percent-specific ^{51}Cr release at an E:T ratio of 1:1. When more than one experiment was done, the mean value is given and the number of experiments is shown in parentheses. When more than two experiments were performed, data are mean ± SD. Lysis of C1R transfectants by these CTL clones was previously reported (32), but is also shown here for comparison. All the CTL, except V26, are from donor M (32).

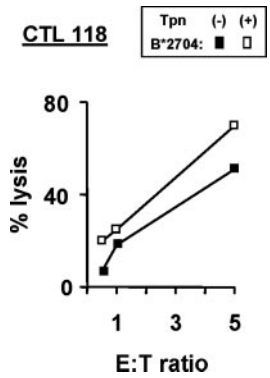

FIGURE 5. Specific cytotoxicity of CTL 118 against Tpn-negative and Tpn-positive .220-B*2704 transfectant cells, at various E:T ratios. A representative experiment, of two independent ones, is shown (see also Table II).

clonal diversity of this set was established from panel analysis with multiple HLA-B27 subtypes and mutants (32), which demonstrated a substantial diversity of clonal reaction patterns. This rules out that our results could be due to restricted heterogeneity of the CTL clones used.

*B*2704 expression and allorecognition are TAP dependent*

Transfection of B*2704 in the TAP-deficient T2 cell line resulted in a moderate surface expression of this allotype at 37°C, which was drastically increased, upon expression of this allotype on T2-TAP transfectant cells (Fig. 6*A*). The amount of B*2704, as judged by quantitative RT-PCR of B*2704 transcripts, was ~2- to 3-fold higher on the TAP-positive T2 transfectants than in the TAP-negative counterparts (Fig. 6*B*). This relatively small difference is very unlikely to account for the much higher surface expression of B*2704 in the TAP-positive cells, confirming the strong TAP dependence of this allotype for peptide supply.

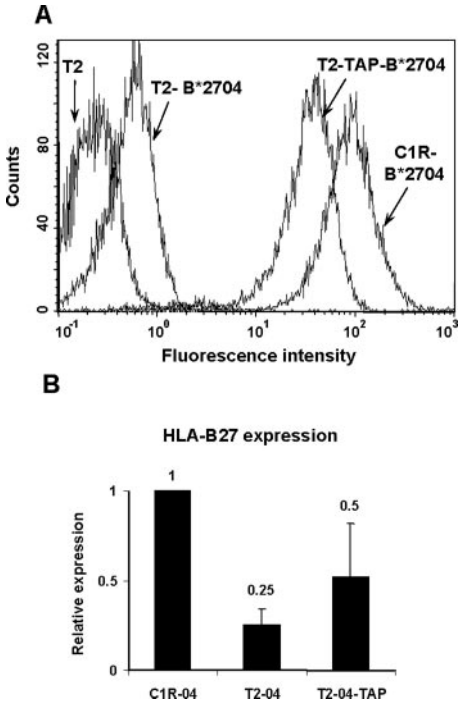

FIGURE 6. Expression of HLA-B*2704 in T2 transfectant cells. *A*, Surface expression of B*2704 on T2 and T2-TAP transfectant cells. The ME1 mAb was used. A representative experiment, of four independent ones, is shown. Mean channel fluorescence values were the following: T2-B*2704, 0.6 ± 1; T2-TAP (not shown), 4 ± 4; T2-TAP-B*2704, 57 ± 24; C1R-B*2704, 72 ± 19. *B*, Expression of B*2704 in C1R, T2, and T2-TAP transfectant cells, assessed by quantitative RT-PCR. Data are mean ± SD of three independent experiments and are relative to the expression of B*2704 in C1R cells.

Table III. *Specific cytotoxicity of anti-B*2704 CTL towards T2-B*2704 and -T2-TAP-B*2704*[a]

CTL	T2-B*2704	T2-B*2704TAP
5	4 (2)	66 (2)
7	20 (2)	46 ± 14 (4)
25	5 (2)	13 ± 4 (3)
45	4	50 (2)
52	0 (2)	36 ± 3 (3)
57	0	45
71	3 ± 5 (3)	32 ± 9 (4)
85	11	42
89	2	28 (2)
95	0	50
96	5 ± 2 (3)	47 ± 14 (6)
106	1 (2)	18 ± 11 (3)
116	6 (2)	40 ± 9 (5)
118	4	38 (2)
131	8 ± 2 (3)	42 ± 16 (5)

[a] Data are expressed as the percent-specific ^{51}Cr release at an E:T ratio of 1:1. When more than one experiment was done, the mean value is given and the number of experiments is shown in parentheses. When more than two experiments were performed, data are mean ± SD.

A total of 15 alloreactive CTL clones elicited against B*2704 from donor M were tested for recognition of T2-B*2704 and T2-TAP-B*2704 targets (Table III). With 1 exception (CTL 7) that showed moderate lysis of the TAP-negative target, all other CTL clones failed to recognize B*2704 in the absence of TAP, but efficiently recognized this allotype on T2-TAP-B*2704 targets. This result indicates that the alloreactive CTL used in these experiments recognize endogenous B*2704 ligands, and that these are generally TAP dependent.

*B*2704 expression, but not allorecognition, is increased by IFN-γ stimulation of C1R cells*

C1R cells express a mixture of constitutive proteasome and immunoproteasome, although the former species is predominant (56). Upon stimulation of C1R-B*2704 cells with IFN-γ, an induction of the immunoproteasome subunit β1i, and a concomitant decrease of the corresponding constitutive subunit β1 was observed by Western blot analysis (Fig. 7*A*). Surface expression of B*2704 was also increased, as established by flow cytometry (Fig. 7*B*). Allorecognition of IFN-γ-treated C1R-B*2704 cells, relative to the same untreated targets, was tested with the 15 peptide-dependent CTL clones used in the previous paragraph (Table IV). IFN-γ-treated targets were lysed with similar efficiency as untreated ones, except for CTL 106. These results suggest that a large majority of the constitutive B*2704-bound peptides recognized by alloreactive CTL are conserved upon immunoproteasome stimulation by IFN-γ.

Discussion

The early stages of HLA-B27 maturation and their consequences on surface expression and stability have attracted recent interest for their putative influence on the pathogenesis of spondyloarthropathies (57). So far, most studies dealing with these issues have been conducted with B*2705. This allotype is strongly dependent on TAP (28), but relatively independent on Tpn (19) for peptide loading, although this chaperone contributes to optimize the B*2705-bound peptide repertoire (16) and introduces significant quantitative

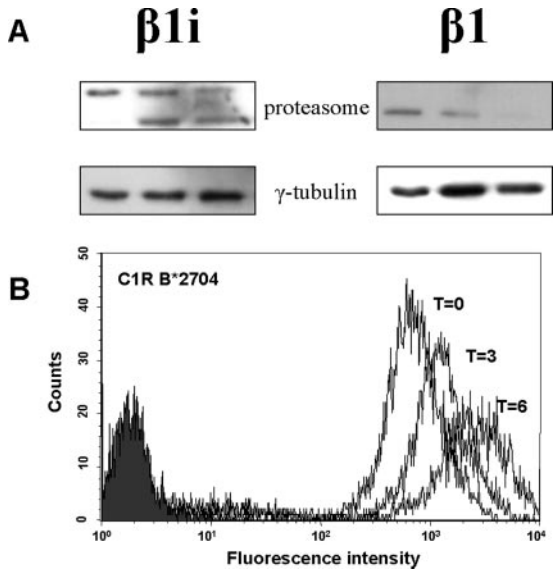

FIGURE 7. Induction of immunoproteasome and surface expression of B*2704 by IFN-γ in C1R cells. *A*, Western blot analysis of the β1i and β1 proteasome subunits at 0, 3, and 6 days after stimulation with IFN-γ (see *Materials and Methods* for details). Blots of tubulin are included as a control. The upper band observed in the β1i blot corresponds to a known cross-reaction of the PW8840 mAb with the β1i precursor. *B*, Flow cytometry analysis of the B*2704 expression on the surface of C1R-B*2704 cells at 0, 3, and 6 days after stimulation with IFN-γ. One experiment, of two independent ones with similar results, is shown.

differences in the bound peptides (15). Moreover, the relatively slow-folding kinetics of B*2705 and its tendency to misfold promotes a larger accumulation of free H chains in the ER than for other class I molecules (24, 26). This correlates with the occurrence of an unfolded protein response in transgenic rats that develop B27-associated disease (58). At the cell surface, covalent forms of β_2m-free H chains, such as homodimers, arise following dissociation of the B*2705 heterodimer upon endosomal recycling (59). Such homodimers may be immunologically relevant because they are recognized by some innate immunity receptors (27, 60).

B*2704 is a prominent subtype in Asian populations and, like B*2705, is strongly associated with AS (30). Two of the three amino acid differences between both allotypes, those at positions 77 and 152, are located in the peptide-binding site. The polymorphism of residue 77 affects peptide-binding specificity, due to its location in the F pocket, which binds the peptidic C-terminal residue (61). The polymorphism of residue 152, which is located in the α2 helix, has a smaller effect on peptide binding (62), but a very strong one on T cell recognition (44). Thus, it was of interest to examine the influence of the B*2704 polymorphism in various aspects of HLA-B27 maturation and peptide loading, including TAP and Tpn dependency, ER accumulation and egress, and immunoproteasome induction, because one or more of these features may be relevant to the role of HLA-B27 in AS.

Table IV. *Specific cytotoxicity of anti-B*2704 CTL towards IFN-γ-treated CIR-B*2704 cells*[a]

CTL	C1R-04	C1R-04 + IFN-γ
5	74 (2)	80 (2)
7	59 ± 14 (4)	66 ± 14 (3)
25	19 ± 3 (3)	19
45	57 (2)	61 (2)
52	50 ± 14 (4)	60 ± 18 (3)
57	65	86
71	43 ± 7 (4)	51 (2)
85	31 ± 5 (3)	40
89	55 (2)	59 (2)
95	40 (2)	39 (2)
96	61 ± 7 (6)	61 ± 15 (4)
106	32 (2)	13 (2)
116	53 ± 9 (5)	62 ± 11 (4)
131	65 ± 6 (5)	69 ± 17 (3)

[a] Data are expressed as the percent-specific ^{51}Cr release at an E:T ratio of 1:1. When more than one experiment was done, the mean value is given and the number of experiments is shown in parentheses. When more than two experiments were performed, data are mean ± SD.

It has been suggested that the strong TAP dependency of B*2705, relative to some other class I molecules may be related to the fact that the N-terminal peptide motifs of HLA-B27, such as R2, are also very good TAP-binding motifs for peptides arising from proteasomal degradation (28, 63). Because B*2704 has the same B pocket as B*2705 and also binds peptides with R2, as well as similar P1 motifs (64), the strong TAP dependency of B*2704 found in our study was not unexpected. Our experiments also confirmed that the B*2704-specific alloreactive CTL were peptide dependent, and that the allospecific peptide epitopes recognized by these CTL were presented by B*2704 only in the presence of TAP.

The Tpn dependency of B*2704 was analogous to that reported for B*2705 (19, 49), in that surface expression of the molecule was significant in the absence of the chaperone, but was nevertheless increased in its presence. Moreover, the heterodimer-to-free H chain ratio was smaller in the absence of Tpn, strongly suggesting that this chaperone, besides increasing surface expression of B*2704, also increases its stability. This is consistent with a role of Tpn in optimizing the peptide cargo of B*2704 toward peptides that bind with higher stability, as reported for B*2705 and other class I molecules (16, 65). The higher amount of free H chains, relative to heterodimer, in the Tpn-deficient .220 cells is likely to arise from cell surface dissociation of suboptimally loaded B*2704 heterodimers, because quality control mechanisms prevent free H chains from exiting the ER (66). However, this dissociation was not reversed to any significant extent by the exogenous addition of a high-affinity B*2704 ligand, suggesting that much of the free H chain in .220 cells might be in irreversible forms, as described for B*2705 (59). Using transient expression in .220 cells it was recently reported that B*2704 is more dependent on Tpn than B*2705 or other B27 subtypes (33). Our results support this view also on stable .220 transfectants, particularly considering that the Tpn-positive B*2704 transfectant expressed less Tpn than the B*2705 counterpart. Increased expression of the B*2704 heterodimer did not result in a concomitant decrease of HC10-reactive material at the cell surface, as observed with B*2705. A possible interpretation of this result is that more suboptimal peptides bind B*2705 than B*2704 and reach the cell surface, in the absence of Tpn. This would be consistent with lower Tpn dependency of the B*2705 heterodimer expression. In the presence of this chaperone, optimized ligands bind to both subtypes and suboptimal peptides are impaired. Because, as mentioned above, HC10-reactive material probably arises at the cell surface following dissociation of suboptimal MHC-peptide complexes, HC10-associated fluorescence would decrease more upon optimization of the B*2705 than the B*2704 peptide cargo. Alloreactive T cell recognition of B*2704 was little affected by the presence of Tpn, strongly suggesting that most of the peptides loaded with Tpn can also be presented by B*2704 in the absence of this chaperone. However, a few CTL clones lysed Tpn-positive cells with increased efficiency. These results are consistent with previous studies on B*2705, showing significant Tpn-dependent quantitative differences in the peptides bound to this allotype (15). Because

activated CTL can recognize minute peptide amounts (67), quantitative differences in the expression of a peptide Ag may not affect target cell recognition and lysis for most of the CTL.

Tpn is a key chaperone in mediating the stable assembly of HLA class I molecules. Presumably, this is due to two interdependent effects. First, its role in maintaining the stability of the peptide-loading complex through interactions with various proteins, including TAP. Second, its role in loading peptides that bind in a more stable way to the class I molecule. The role of Tpn in mediating the efficient folding of the B*2704 heterodimer was well apparent from the significantly increased accumulation of unfolded H chain in the absence of the chaperone. However, it was remarkable that the kinetics of exiting the ER of the mature B*2704 protein was little affected by Tpn. This result strongly suggests that Tpn controls the amount of B*2704 that folds into the mature heterodimer-peptide complexes, but not, or little, its rate of egress from the ER. This is consistent with the idea that all that is required for the HLA class I molecule to leave the ER is to form a complex with peptide beyond a certain stability threshold (49). The efficiency with which these complexes are formed is Tpn dependent but, once formed Tpn does not play a significant role in their egress. B*2706 showed a different behavior. Even in the absence of Tpn, it did not accumulate in the ER and its export rate was much faster than for B*2704. Although Tpn-positive .220 transfectants were not available, precluding a direct analysis of the effect of Tpn on B*2706 in this cell line, the maturation of this allotype was clearly less dependent on this chaperone, relative to B*2704 or B*2705. This was confirmed by the similar behavior of B*2706 in the Tpn-positive C1R-B*2706 (our unpublished observations; B. Galocha and J. A. López de Castro, manuscript in preparation), and 721.221 cells (33). Because B*2706 has D114, our results do not support that this single residue determines Tpn dependency, as previously claimed (68). Presumably, the concomitant D116Y change in this subtype compensates for the effect of D114 in HLA-B27.

In the TAP and Tpn-proficient C1R cells, IFN-γ influenced both the induction of immunoproteasome and an increase in surface expression of B*2704. However, in general, T cell allorecognition of this allotype was not affected. This suggests that immunoproteasome induction has a limited qualitative effect on the B*2704-bound peptide repertoire without excluding, neither quantitative changes in the expression of peptide epitopes nor occasional destruction of some of them, as has been previously observed (69). However, the lower recognition of B*2704 upon IFN-γ by one CTL clone in our study, does not necessarily imply destruction of the epitope by the immunoproteasome, because it may result from other induced changes in the cell, such as for instance, lower expression of the parental protein for the corresponding epitope.

Overall, the B*2704 behavior concerning TAP, Tpn, and proteasome dependency for maturation, surface expression, peptide presentation, and T cell recognition seems to be qualitatively similar to B*2705, without excluding quantitative differences, particularly on the influence of Tpn on surface expression. As already noted (33), subtype differences in their Tpn dependency for surface expression do not correlate with association to AS. However, the similar behavior of B*2704 and B*2705 in the extent of folding and export rate form the ER as a function of Tpn, and their differential behavior with B*2706 in these features is compatible with a role of the maturation properties of HLA-B27 subtypes in determining susceptibility to AS, in line with the predictions of the "misfolding" hypothesis for the pathogenesis of this disease (25). Thus, both B*2704 and B*2705 have an intrinsic tendency to misfold and accumulate in the ER, which is revealed specially in the absence of Tpn, and a relatively slow export rate. In contrast, B*2706, which is not associated to AS, folds efficiently and is exported quickly in the absence of Tpn, so that this subtype has an intrinsic folding capacity that is higher than the AS-associated subtypes. As predicted by the "misfolding" hypothesis, any conditions that may exacerbate the intrinsic tendency of B*2704 and B*2705 to misfold might elicit an unfolding protein response and trigger inflammation. Such conditions would presumably fail to be inflammatory in B*2706 individuals, leading to protection from disease. However, it must be noted that B*2709, which is also not associated with AS (70, 71) matures more like B*2704 and B*2705 than like B*2706, at least in the presence of Tpn (33). Thus, despite the differential behavior of B*2706 in folding and export rate, the relevance of these features for the pathogenesis of AS and for the differential association of HLA-B27 subtypes to this disease remains unclear.

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Disclosures

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