HLA-B27 Misfolding Is Associated with Aberrant Intermolecular Disulfide Bond Formation (Dimerization) in the Endoplasmic Reticulum*

The class I protein HLA-B27 confers susceptibility to inflammatory arthritis in humans and when overexpressed in rodents for reasons that remain unclear. We demonstrated previously that HLA-B27 heavy chains (HC) undergo endoplasmic reticulum (ER)-associated degradation. We report here that HLA-B27 HC also forms two types of aberrant disulfide-linked complexes (dimers) during the folding and assembly process that can be distinguished by conformation-sensitive antibodies W6/32 and HC10. HC10-reactive dimers form immediately after HC synthesis in the ER and constitute at least 25% of the HC pool, whereas W6/32-reactive dimers appear several hours later and represent less than 10% of the folded HC. HC10-reactive dimers accumulate in the absence of tapasin or β2-microglobulin, whereas W6/32-reactive dimers are not detected. Efficient formation of W6/32-reactive dimers appears to depend on the transporter associated with antigen processing, tapasin, and β2-microglobulin. The unpaired Cys67 and residues at the base of the B pocket that dramatically impair HLA-B27 HC folding are critical for the formation of HC10-reactive ER dimers. Although certain other alleles also form dimers late in the assembly pathway, ER dimerization of HLA-B27 may be unique. These results demonstrate that residues comprising the HLA-B27 B-pocket result in aberrant HC folding and disulfide bond formation, and thus confer unusual properties on this molecule that are unrelated to peptide selection per se, yet may be important in disease pathogenesis.

The proper folding and assembly of proteins in the endoplasmic reticulum (ER)† is dependent on a number of chaperones, and is subject to stringent quality control measures to ensure that improperly folded proteins are destroyed (1). For major histocompatibility complex (MHC) class I complexes, newly synthesized unfolded heavy chains (HC) bind to calnexin, with subsequent folding, release, and calreticulin binding coincident with β2-microglobulin (β2m) association (2). Further retention of partially folded HC/β2m heterodimers is mediated by tapasin, an MHC class I-specific chaperone which forms a bridge between HC and TAP (transporter associated with antigen processing) in the peptide loading complex. Tapasin-mediated retention serves to optimize peptide loading and thus stability, so that complexes can traffic to the cell surface and display peptides without dissociating prematurely. An important component of HC folding and assembly is the formation of correct intrachain disulfide bonds (3), as evidenced by mutations in critical Cys residues that interfere with class I expression and antigen presentation (4). For MHC class I proteins this appears to be mediated by ERp57, a thiol oxidoreductase that catalyzes disulfide bond formation and rearrangement. ERp57 has been found associated with calnexin and calreticulin (5), and is also part of the peptide-loading complex (6–8) where it is linked to tapasin via a disulfide bond (9).

When class I HC are synthesized in the absence of β2m or peptide, they misfold and are dislocated from the ER into the cytosol where they are degraded in a process known as ER-associated degradation (10). When certain mouse class I alleles are expressed in β2m-deficient cells, misfolding is associated with the formation of HC-β2m homodimers in the ER via an unpaired Cys in the cytoplasmic tail (11). In these studies HC dimerization was also observed in cells expressing β2m, but only after β2m had dissociated from previously assembled complexes. This led to the suggestion that HC dimerization might be a mechanism by which dysfunctional molecules are removed (11).

HLA-B27 is an unusual allele in that it misfolds even in the presence of a normal supply of β2m and peptide (12). This characteristic is related to the composition of its B pocket, a region of the peptide-binding groove that plays a dominant role in peptide selection (13, 14), but also has a dramatic influence on HC folding rate (12). The rim of the B pocket also contains the unpaired reactive Cys residue (Cys67) (15, 16), which is critical for HC folding (12). The rim of the B pocket is important for HC-HC homodimers in the ER via an unpaired Cys in the cytoplasmic tail (11). In these studies HC dimerization was also observed in cells expressing β2m, but only after β2m had dissociated from previously assembled complexes. This led to the suggestion that HC dimerization might be a mechanism by which dysfunctional molecules are removed (11).

* This work was supported in part by the Schmidlapp Foundation, the Arthritis Foundation (to D. J. K. and R. A. C.), National Institutes of Health Grants R01 AR-46177 and R60 AR-44059 (to R. A. C.), and grants from the Sonnenfeld-Stiftung, Berlin (to B. U.-Z. and A. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; β2m, β2-microglobulin; HLA, human leukocyte antigen; HC, heavy chain; TAP, transporter associated with antigen processing; hMW, high molecular weight; PBS, phosphate-buffered saline.

This paper is available online at http://www.jbc.org

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Received for publication, October 26, 2001, and in revised form, April 2, 2002

Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M110336200
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HLA-B27 is highly associated with susceptibility to ankylosing spondylitis and other human spondyloarthropathies (20), and can cause spondyloarthropathy-like inflammatory conditions when overexpressed in rodents (21, 22). Several hypotheses have been proposed to explain these findings, but as yet the mechanism is unclear. Whereas arthritogenic peptide presentation has been suspected for some time, there is limited support for this hypothesis (23), and in instances where autoreactivity has been found, it is not clear whether this is either specific for, or initiated by, HLA-B27 (24, 25). The results presented here indicate that aberrant misfolding of HLA-B27 in cells with an intact ER. The folding of HLA-B27 may be unique to this allele, and could be involved in the pathogenesis of spondyloarthropathies.

EXPERIMENTAL PROCEDURES

DNA and Cell Lines—B27.A23 and B27.E45M were constructed from a genomic clone of HLA-B*2705 by site-directed mutagenesis (Altered Sites, Promega, Madison, WI). B27.A23 has three amino acid substitutions: H9F (F for H at position 9), T24A, and E45M. B27.C67A was kindly provided by Joel Taurog, and was also produced from genomic scriptase-PCR using oligonucleotides designed from the published sequence (33), and is the allele with Thr at position 240 of the mature protein (34). HF and BL are B-lymphoblastoid cell lines. HF express HLA-A2, -A3, -B7, -B27, and -Cw4; BL express -B8 and -B27, but HLA-A alleles are not expressed. T5-1 is a B cell line expressing HLA-A1, -A2, -B8, -B27, -B5, and -Cw1. T5-1 is a B cell line expressing HLA-A1, -A2, -B8, -B27, -B5, and -Cw1.

Immunoprecipitations—Cells were rinsed in ice-cold PBS (pH 7.2), then suspended at 5 × 10^7 cells in 0.5 ml of cell suspension was added 400 μl of freshly made Sulo-NHS-biotin (Pierce) solution (1.5 mg/ml in PBS with 5% FCS) and this solution was incubated for 30 min at 4°C with rocking. Unconjugated reagent was removed with several washes using ice-cold PBS, and the labeled cells were subsequently lysed and used for immunoprecipitations.

Immunoprecipitations—Immunoprecipitations were performed in the following manner unless otherwise stated. Cell lysates were pre-cleared with washed formalin-fixed Staphylococcus aureus (Sigma), and incubated with purified monomeric antibody (15 μg per 2 × 10^7 cells) for 0.5 ml at 4°C, followed by the addition of Protein A-Sepharose (100 μl of a 50 mg/ml suspension in PLB (Sigma)) and another hour at 4°C. Protein A-Sepharose pellets were washed consecutively with PLB supplemented to 0.1% SDS and 1% bovine serum albumin, a 10-fold dilution of PLB supplemented to 80 mM NaCl, and PLB alone, and stored at 20°C until electrophoresis.

Enzymatic Digestions—Proteins were eluted from immunoprecipitates by boiling for 5 min in 0.1% SDS (Bio-Rad), then digested overnight at 37°C in the absence of reducing agents. For endoglycosidase H (Roche Molecular Diagnostics, Mannheim, Germany), 2 milliunits in 75 mM sodium citrate (pH 5.5) were used; for N-glycosidase (Glyko, Novato, CA), 10 milliunits in 20 mM sodium phosphate (pH 7.5) were used, and for endoproteinase Glu-C, citrate buffer alone was used.

Gel Electrophoresis and Phosphorimaging—Isoelectric focusing (IEF) and SDS-PAGE (10.5% gels) were performed as described previously (12). For reducing and nonreducing SDS-PAGE, samples were boiled for 5 min in an equal volume of 2 × sample buffer with or without dithiothreitol (200 mM), respectively, prior to electrophoresis. For phosphorimaging analysis, gels were fixed in 10% acetic acid for 30 min, then dried. Dried gels were exposed to PhosphorImager plates for 72 h, and 35S-labeled proteins were visualized and quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

HLA-B27 HC Form W6/32 and HC10-reactive Disulfide-linked Complexes in Cells—To determine whether HLA-B27...
HC form aberrant disulfide bound complexes in cells with an intact class I assembly pathway, class I molecules were immunoprecipitated from C1R.B27, separated on SDS-PAGE under nonreducing and reducing conditions, and visualized by immunoblotting. High molecular weight (hMW) HC complexes are seen migrating at ~90 kDa in addition to monomers at ~45 kDa (Fig. 1A, NRRed). The absence of the hMW bands in untransfected C1R and their elimination by sample reduction prior to electrophoresis (Fig. 1A, red) indicates they are indeed HLA-B27 HC. These hMW complexes are also seen when cell lysates are applied immediately to SDS-PAGE, indicating that they are not an artifact of immunoprecipitation (data not shown). To prevent post-lysis disulfide bond formation which can occur with HLA class I molecules, particularly during prolonged purification protocols (44, 45), iodoacetamide is included in the cell lysis and sample buffers for all experiments reported here.

W6/32 and HC10 recognize largely distinct populations of HLA class I molecules (38, 40). W6/32 is conformation dependent, recognizing folded complexes containing peptide. In contrast, HC10 recognizes free HC not associated with β2m. Thus we refer to W6/32 and HC10-reactive HC as folded and unfolded, respectively, although the degree to which HC10-reactive HC are unfolded is not known (see “Discussion”). The heterogeneity of the hMW complexes recognized by HC10 in comparison to W6/32 (Fig. 1A) together with sequential immunoprecipitations where W6/32-or HC10-reactive material remains after pre-clearing with the reciprocal antibody (Fig. 1B), reveals that these complexes also contain distinct groups of HLA-B27 molecules. 5H7, which recognizes a folded α3 epitope, does not react with the more heterogeneous HC10-reactive material (Fig. 1A), raising the possibility that the α3 domain is not properly folded or may be blocked by another protein interacting with free HC. Based on the apparent $M_r$ (~90,000) of the homogeneous band immunoprecipitated by W6/32 and 5H7, its disappearance with sample reduction, and the previous report of HLA-B27 dimerization in vitro (18), we conclude that it is a HC-HC dimer linked by a disulfide bond. Consistent with this, purified W6/32-reactive material reveals only two protein bands (HC and β2m) following sample reduction (data not shown). However, we have not completely ruled out the possibility that the hMW complexes, particularly those recognized by HC10, might contain an additional protein (or proteins) (see “Discussion”).

A small percentage of cell surface HLA-B27 complexes are recognized by the monoclonal antibody MARB4. When purified from whole cell lysates, these were found to contain peptides of a much broader size range than the canonical 8–11 residues, including peptides up to 33 amino acids in length (43). Immunoprecipitations with MARB4 reveal that this antibody recognizes dimers as well as monomers (Fig. 1C). It is worth noting that the relative intensity of the 90-kDa band compared with the monomer is greater with MARB4 than for other monoclonal antibodies, suggesting that a higher proportion of MARB4-reactive molecules are dimers. The smaller quantity of HC immunoprecipitated with MARB4 (per cell equivalent) is consistent with the lower expression of MARB4-reactive molecules (43) and data not shown). The immunoreactive material in the mock lanes (no cell lysate) is a result of cross-reactivity with the rabbit anti-mouse IgG used to enhance immunoprecipitation. MARB4-reactive dimers can be immunoprecipitated without addition of this cross-linking antibody if more cells are used, whereas MARB4 immunoprecipitations of untransfected C1R reveal no dimer band (data not shown). Recognition of HLA-B27 dimers by MARB4 raises the possibility that some may contain long peptides, although this will need to be addressed experimentally.

Taken together, these data indicate that the W6/32-reactive HLA-B27 HC dimers reported previously to form in the absence of β2m or peptide (18) form in cells with an intact class I
assembly pathway. Furthermore, there appears to be a distinct pool of dimers that are reactive with HC10 but not W6/32, and thus are unfolded or possibly misfolded.

**Kinetics of HLA-B27 HC Dimerization**—To assess the kinetics of HLA-B27 dimerization, cells were pulsed with $[^{35}S] Met/Cys$, then chased in media containing excess nonradioactive Met/Cys, and lysed in buffer containing Triton X-100 to dissociate HC from weakly interacting proteins including the pep-tide loading complex (46). HC10-reactive dimers are present after a 30-min labeling period (0 h chase), and account for $\sim 30-40\%$ of the total HC10-reactive HC (Fig. 2A) based on PhosphorImager quantitation (data not shown). It should be noted that this is a minimum estimate, as some of the HC monomer band in C1R.B27 derives from HLA-C (35), whereas this allele does not contribute to the dimer bands (Fig. 2A, C1R lane). Dimers and monomers decay at approximately the same rate during the 4-h chase, and are initially sensitive to Endo H (Fig. 2B). However, after longer chase periods (>6 h), HC10-resistant HC10-reactive dimers can be detected (data not shown). There is a faint band migrating near the bottom of the hMW HC (4 h lane, bracket) that is not seen in untransfected C1R and remains after sample reduction (1.5 h lane, red). This band is not HC as it is not reactive with anti-HC antibody in immunoblots (Fig. 1A). It has been tentatively identified as the ER chaperone BiP/GRP78. The rapid formation and initial sensitivity of HC10-reactive HLA-B27 dimers to Endo H indicates they form in the ER shortly after HC synthesis.

W6/32-reactive dimers were not detected during the time course shown in Fig. 2A (data not shown), so cells were labeled and chased for a longer period to enhance detection. Dimers representing up to $\sim 10\%$ of the total W6/32-precipitable HC were detected after a 12-h labeling period (Fig. 2C) that decayed with kinetics similar to folded monomer HC. Folded dimers were found to be Endo H-resistant in immunoblotting experiments (Fig. 2B), indicating they have been sialylated. Both folded and unfolded dimers can be labeled with a cell-permeable biotinylating reagent (Fig. 2D), indicating that at least some are on the cell surface. It should be noted that W6/32 has been shown to react poorly with biotinylated HLA-B27 (47) (and Fig. 2D) so that 5H7 was used for this experiment. These results indicate that the kinetics of formation of HC10- and W6/32-reactive HC dimers are dramatically different, and thus provide further evidence that they represent distinct pools of aberrant disulfide-linked HLA-B27 complexes.

**The Role of TAP, Tapasin, and $\beta_2$-Microglobulin in the Formation of HLA-B27 Dimers**—To determine whether the presence of an intact peptide loading complex influences HLA-B27 dimerization, we examined HLA-B27 expression in TAP1/TAP2-deficient (T2), tapasin-deficient (721.220), and $\beta_2$m-deficient cells. Neither W6/32- nor HC10-reactive dimers are detected in immunoprecipitates from T2 cells that have been transfected with HLA-B27 (T2.B27) or cDNAs encoding TAP1

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Fig. 2. Kinetics of formation of HC-10- and W6/32-reactive HLA-B27 dimers. A, cells were labeled with $[^{35}S] Met/Cys$ for 30 min, then chased for $0-4$ h. Immunoprecipitations were performed with HC10, and material was electrophoresed on SDS-PAGE under nonreducing conditions, except for the 1.5-h time point which included a sample run under reducing conditions (Red). Phosphorimaging was used to visualize radioactive material. hMW HC complexes are indicated by the bracket, and monomer HC by the arrow. B, top, immunoprecipitated HC10-reactive material at 1.5 h of chase was divided into three aliquots, and incubated in the absence of reducing agent with Endo H, N-glycanase as a positive control, or buffer alone (M, mock) as described under “Experimental Procedures.” Radioactive material was visualized by phosphorimaging. Bottom, immunoprecipitated W6/32-reactive material from nonlabeled C1R.B27 cells was digested as described above, separated by SDS-PAGE under nonreducing conditions, and visualized by immunoblotting with 3B10.7. C, cells were labeled for 12 h, and chased for up to 24 h. Immunoprecipitations were performed with W6/32. Arrows indicate the position of dimers and monomer HC. D, C1R.B27 cells were biotinylated, then lysed and immunoprecipitations performed with the indicated antibodies. M indicates biotinylated cells processed in parallel, but without any primary antibody added. Each lane in A, C, and D represents material recovered from $10^7$ cells.
and TAP2 (T2.TAP) (Fig. 3A). However, when HLA-B27 is expressed in T2.TAP, a faint W6/32-reactive dimer band is detected, whereas HC10-reactive dimers are not seen. This observation was confirmed in independent transfectants using FLAG-tagged HLA-B27 (data not shown). It should be noted that the overall recovery of HC from these transfectants reflects the effect of TAP on the formation of stable peptide-loaded complexes. We considered the possibility that loading material derived from more cells might allow the detection of dimers. Although no W6/32-reactive dimer band could be detected even when 4 times as many cells were used, HC10-reactive material from T2.B27 and T2.TAP.B27 became apparent (data not shown).

The TAP dependence for formation of W6/32-reactive dimers is in contrast to a previous report (18), so we sought to confirm these findings in a separate cell line. T5-1 cells which are a B-lymphoblastoid line expressing HLA-B27 as well as -A1, -A2, and -B8 alleles were examined (48). This cell line has been mutagenized, and derivatives lacking MHC genes have been selected in a fashion analogous to the generation of T2 (36). 8.1.6 are deficient in the MHC region encoding TAP1 and TAP2 on one chromosome, whereas 5.2.4 lack both copies of TAP1/TAP2 as well as HLA-A1 and -B8, but still express HLA-A2 and -B27. W6/32- and HC10-reactive dimers are found in T5-1 and 8.1.6, but not 5.2.4 (Fig. 3B). However, similar to T2.B27, HC10-reactive dimers can be detected even when 5.2.4 cells are used (data not shown). ME.1 immunoprecipitations proved that the folded dimers are indeed HLA-B27 HC (data not shown), because this antibody does not recognize the other alleles expressed in T5-1. The results with W6/32 are consistent with those obtained in T2 transfectants, and suggest that formation of folded HLA-B27 dimers requires the transporter associated with antigen processing. HC10-reactive dimers are more readily detected in T5-1 and 8.1.6 cells for reasons that are unclear.

When HLA-B27 is expressed in 721.220 cells (.220.B27) which express only small amounts of a truncated form of tapasin (34), W6/32-reactive dimers are not detected, but in contrast to T2, HC10-reactive dimers accumulate (Fig. 3C). Re-expression of tapasin (.220.Tsn.B27) enables folded dimers to be recovered and substantially reduces the pool of unfolded dimers (Fig. 3C). As expected it also enhances the recovery of W6/32-reactive monomers (i.e., single-HC/peptide/β2m complexes), and reduces the accumulation of HC10-reactive material. The proportion of dimerized HLA-B27 HC appears to be greater in cells lacking tapasin (Fig. 3C) than in TAP-deficient cells (Fig. 3, A and B). TAP-transported peptides may actually play a role in the formation and/or stabilization of HC10-reactive dimers.

Expression of HLA-B27 HC in the absence of β2m eliminates the formation of W6/32-reactive dimers, and only a very faint monomer band can be detected (Fig. 3D). The absence of folded monomers is expected because β2m is critical for proper HC folding and formation of the epitope recognized by W6/32. In striking contrast, HC10-reactive dimers and monomers are recovered from β2m-deficient cells in proportions similar to
what is found when HLA-B27 is expressed in the absence of tapasin (Fig. 3C). Taken together, these results suggest that β2m, tapasin, and TAP are all required for the formation of W6/32-reactive HLA-B27 dimers, and at least for β2m and tapasin, unfolded dimers tend to accumulate in their absence.

ER Retention and Cys67 Contribute to HLA-B27 Dimerization—Previously we demonstrated that substituting 6 amino acids in HLA-B27 to create an HLA-A2-like B pocket (referred to as B27.A2B) prevents misfolding and ER-associated degradation of HC (12). To determine whether these structural changes affect HC dimerization, we examined B27.A2B and three other mutants with B pocket substitutions. Replacing the entire B pocket (B27.A2B) prevents the accumulation of both unfolded and folded dimers (Fig. 4A). This can also be achieved by replacing only Cys67 with Ala (B27.C67A), which confirms previous results showing that this residue is necessary for HLA-B27 HC dimerization in vitro (18). Surprisingly, substituting residues at the base of the B pocket without replacing Cys67 also affects dimerization. For B27.E45M no dimers are detected with immunoblotting, whereas for B27.A23 a faint dimer band can be seen.

To further investigate why dimerization is affected in the mutants that still contain Cys67 HC folding efficiency was compared. C1R transfectants were pulsed for 5 min with [35S]Met/Cys and chased for up to 2 h. At each time point, W6/32- and HC10-reactive HC was immunoprecipitated sequentially from cell lysates, then visualized and quantitated following separation on IEF gels run under reducing conditions. Gel regions from a representative experiment comparing HLA-B27 with B27.E45M, along with quantitative results averaged from two experiments, show that the conversion of HC from an HC10- to W6/32-reactive state is much more rapid for B27.E45M than for HLA-B27 (Fig. 4B). This analysis was applied to each B pocket mutant, and the time required for 75% of newly synthesized HC to fold (become W6/32-reactive) was determined from each graph. Results averaged from two experiments are depicted in Fig. 4C. The data showing HLA-B27 and B27.A2B folding have been reported previously (12) but are included for purposes of comparison. B27.A23 and B27.E45M fold dramatically faster than wild type HLA-B27 or B27.C67A, and are similar to B27.A2B. The rapid folding molecules have in common the substitution of Met for Glu at position 45 at the base of the B pocket. The Glu substitution removes an acidic residue that is expected to be predominantly negatively charged at neutral pH, and is neutralized when HLA-B27 binds a peptide with Arg at P2 (49). These results indicate that at least in the context of the HLA-B27, Glu45 has a dramatic effect on HC folding rate, increasing the amount of time in which HC exists in the ER in an unfolded state.

Although immunoblotting experiments do not reveal dimers in cells expressing B27.E45M (Fig. 4), it was possible that they might form but fail to accumulate because of rapid degradation.
To test this, cells expressing HLA-B27 and B27.E45M were labeled with [35S]Met/Cys for 2 h, then chased for up to 19 h, and the presence of unfolded and folded dimers analyzed by nonreducing conditions. Prominent unfolded hMW HC bands (Fig. 5) (top panel, left bracket), whereas only a faint band is observed for B27.E45M (bottom panel). A band representing folded W6/32-reactive dimers that contains ~7% of the total labeled HC is seen after 6 h of chase for HLA-B27, whereas this is not apparent in B27.E45M immunoprecipitates. We have used this assay to examine B27.A23 and find that like B27.E45M, it does not form HC10-reactive dimers in the ER, however, W6/32-reactive dimers can be detected after 6 h of chase (data not shown). Considered together, the results shown in Figs. 4 and 5 demonstrate that there are two characteristics contributing to the formation of HLA-B27 dimers in the ER. Cys67 is necessary but not sufficient, as B27.E45M does not dimerize. Impaired folding is necessary but not sufficient, as B27.C67A does not dimerize. It appears that the formation of aberrant disulfide bonds between HLA-B27 HC in the ER results from the combination of impaired folding with ER retention of HC in the presence of the unpaired Cys67.

Dimer Formation in B-LCL and by Other HLA Class I Molecules—In addition to T5-1, we find dimers in other B cell lines (Fig. 6, HF and BL). Because these cells express other class I alleles in addition to HLA-B27, we confirmed that the dimers can be immunoprecipitated by ME.1 (data not shown). However, because BL express HLA-B8 and HF express HLA-B7, the latter of which is recognized by ME.1, we tested these alleles along with HLA-A2 and -B53 in C1R cells. Whereas we find no evidence of dimers for HLA-B8, -A2, and -B53, a dimer-sized band can be detected with HLA-B7 (Fig. 6). Thus, the dimer band in HF could represent HLA-B27 or -B7 (or both). We have tested several additional cell lines including Jurkat, U937, and HeLa, and find no evidence of HLA class I HC dimerization (data not shown). Jurkat express HLA-A3, A9, B16, and B35 (50), although other references indicate they express HLA-B7 (51, 52). U937 express HLA-B7, -A2, and -B53 along with HLA-A2 and -B53 in C1R cells. Whereas we find no evidence of dimers for HLA-B8, -A2, and -B53, a dimer-sized band can be detected with HLA-B7 (Fig. 6). Thus, the dimer band in HF could represent HLA-B27 or -B7 (or both). U937 express HLA-A3, A9, B16, and B35 (50), although other references indicate they express HLA-B7 (51, 52). U937 express HLA-A3, -A9, -B16, and -B35 (50). Thus, whereas class I HC dimerization is not unique to HLA-B27, it does not appear to be a generalized phenomenon.

HLA-B7 does not contain an unpaired Cys in its extracellular domain, so we considered the possibility that it might differ from HLA-B27 in the formation of dimers. Indeed, when HLA-B7 HC are labeled and chased in an experiment identical to that shown in Fig. 5, less than 5% of total labeled HC appear in the hMW region of the HC10 gel (Fig. 6B). However, folded W6/32-reactive dimers appear with kinetics similar to HLA-B27 and the mutant B27.A23, and constitute ~8% of the total HC at 6 h of chase. It is also worth noting that approximately two-thirds of HLA-B7 HC synthesized in the 2-h labeling period are folded, which is similar to B27.E45M and dramatically different from HLA-B27 where only one-third (34%) are folded (Fig. 5). The formation of W6/32-reactive dimers late in the assembly process (perhaps at the cell surface), by HLA-B7 and B27.A23 that do not first form HC10-reactive dimers in the ER, emphasizes that ER dimerization is not required.

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**Fig. 5. Rapid folding prevents formation of HLA-B27 dimers.** C1R expressing HLA-B27 and B27.E45M were labeled for 2 h with [35S]Met/Cys, then chased for up to 19 h. At each time point cell lysates were divided into equal aliquots, and immunoprecipitations were performed with HC10 and W6/32. Samples were separated by SDS-PAGE under nonreducing conditions, and visualized by phosphorimaging. Background-subtracted radioactivity in monomer (Mon) and dimer (hMW) bands was quantitated, and is expressed below each gel as a percentage of the total HC (W6/32 and HC10). Brackets and arrows indicate the regions that were quantitated. Note that for purposes of quantitation only the upper two hMW HC bands were assessed, as the lowest band does not always separate from a co-precipitating protein (see Fig. 2A). Asterisks indicate nonspecific bands. – indicates that radioactivity was <5% of total.

**Fig. 6. Specificity of dimer formation.** A. HLA class I complexes were immunoprecipitated with W6/32 from B lymphoblastoid cell lines (B-LCL: HF and BL) expressing HLA-B27 as well as other alleles, and from C1R transfected with different HLA alleles, separated by SDS-PAGE under nonreducing conditions, and HC visualized by immunoblotting with 3B10.7. Each lane represents material immunoprecipitated from 2 × 10⁷ cells. B, C1R.B7 dimerization was analyzed exactly as described in the legend to Fig. 5 for HLA-B27 and B27.E45M. – indicates that radioactivity was <5% of total.
Our results indicate that HLA-B27 HC form two types of aberrant disulfide-linked complexes (dimers) that are distinguishable by several criteria, including kinetics and site of formation, migration on nonreducing SDS-PAGE, dependence on β2m, TAP, and tapasin, and recognition by monoclonal antibodies HC10 and W6/32. HC10 recognizes newly synthesized HC before they are completely folded and have bound β2m (54), free HC on the cell surface that have lost β2m (55), and completely denatured HC separated on SDS-polyacrylamide gels (40). In contrast, W6/32 recognizes a conformational epitope on completely denatured HC separated on SDS-polyacrylamide gels somewhat unfolded relative to W6/32-reactive monomeric HC, epitopes. Furthermore, HC in W6/32-reactive dimers may be possibility that a small proportion of dimers may contain both pools of HLA-B27 HC dimers, however, we cannot rule out the possibility that this can occur with HLA-B7 HC consistent with a previous report (60). However, there are important differences in that the majority of HLA-B27 HC dimerization occurs in the ER, and ER dimerization of mouse alleles was observed only when β2m was absent, a condition that induces HC misfolding (10). In the presence of β2m, mouse HC dimerization occurred in a post-ER compartment and was associated with loss of β2m. Rapidly folding molecules such as HLA-B7 and the HLA-B27 mutant B27.A23 appear to be more similar to mouse class I in that ER dimerization does not occur in cells expressing β2m. Neither HLA-B7 nor the mouse alleles have unpaired Cys residues in their extracellular domain, and indeed mouse HC dimerization appeared to occur through a cytoplasmic tail Cys (11). Taken together these results suggest that the dimerization of HLA-B27 HC in the ER in the presence of an intact class I assembly pathway fundamentally distinguishes HLA-B27 dimerization from what has been observed with other alleles, and may contribute to the unusual tendency of this allele to misfold (12). Our mutagenesis studies identify two characteristics of the HC that are critical for ER dimerization, Cys67 and impaired folding with ER retention. The requirement for Cys67 is consistent with previous data suggesting that this residue forms a disulfide link between two HC (18). The importance of impaired HC folding is best exemplified by B27.E45M, which contains a Cys67 but folds very rapidly and fails to form disulfide-linked HC complexes. These data are consistent with a model where impaired folding of newly synthesized HLA-B27 HC leaves the Cys67 exposed, and thus prone to form aberrant links to other HC particularly in the oxidizing environment of the ER. Folded dimers could emerge from the unfolded dimer pool, and/or form later after folded monomers (HC/peptide/β2m) lose β2m during transport or at the cell surface. The tendency of B27.A23, HLA-B7, and mouse alleles to display the late dimerization phenotype without significant ER dimerization indicates that this pool does not have to arise from ER dimers. B pocket mutants like B27.E45M that fold rapidly and avoid ER dimerization might also avoid late dimerization if the mutation results in greater HC/peptide-β2m complex stability compared with wild type HLA-B27 or the B27.A23 mutant.

The reduced accumulation of unfolded HLA-B27 dimers in the absence of TAP (T2.B27 and 5.2.4 cells), and their prominence in the absence of tapasin (220.B27 cells) or β2m, suggests that TAP-transported peptides may contribute to their formation and/or stabilization, whereas tapasin facilitates their elimination. In support of this, it is clear from several

DISCUSSION

system with microsomes (58), and in the other using a highly sensitive immunoblotting technique with 125I-labeled anti-ER60 (ERP57) (59). In the latter studies, HC-ERP57 complexes were estimated to comprise <1% of the total HC pool, consistent with the notion that they are transient folding intermediates. We have immunoblotted nonreduced HC10 immunoprecipitates with anti-ERP57 antibodies but do not detect ERP57 in any of the three hMW HC bands. (Blots for calreticulin (~54 kDa) and tapasin (~48 kDa) were also negative.) Because the HC10-reactive dimer complexes we observe constitute a large portion of the newly synthesized HC pool, decay slowly over several hours, do not appear to contain ERP57, and depend on Cys67 and impaired folding to form, we conclude that they are not likely to represent intermediates in a normal folding process. It seems more likely that they represent HC-HC homodimers of HLA-B27 with variable disulfide bonds, although further experiments will be necessary to determine their precise composition.

Class I HC dimerization is not a generalized phenomenon, yet it is not unique to HLA-B27. Mouse class I alleles H-2Ld, D4, and Db have been shown to dimerize (11), and we demonstrate that this can occur with HLA-B7 HC consistent with a previous report (60). However, there are important differences in that the majority of HLA-B27 HC dimerization occurs in the ER, and ER dimerization of mouse alleles was observed only when β2m was absent, a condition that induces HC misfolding (10). In the presence of β2m, mouse HC dimerization occurred in a post-ER compartment and was associated with loss of β2m. Rapidly folding molecules such as HLA-B7 and the HLA-B27 mutant B27.A23 appear to be more similar to mouse class I in that ER dimerization does not occur in cells expressing β2m. Neither HLA-B7 nor the mouse alleles have unpaired Cys residues in their extracellular domain, and indeed mouse HC dimerization appeared to occur through a cytoplasmic tail Cys (11). Taken together these results suggest that the dimerization of HLA-B27 HC in the ER in the presence of an intact class I assembly pathway fundamentally distinguishes HLA-B27 dimerization from what has been observed with other alleles, and may contribute to the unusual tendency of this allele to misfold (12). Our mutagenesis studies identify two characteristics of the HC that are critical for ER dimerization, Cys67 and impaired folding with ER retention. The requirement for Cys67 is consistent with previous data suggesting that this residue forms a disulfide link between two HC (18). The importance of impaired HC folding is best exemplified by B27.E45M, which contains a Cys67 but folds very rapidly and fails to form disulfide-linked HC complexes. These data are consistent with a model where impaired folding of newly synthesized HLA-B27 HC leaves the Cys67 exposed, and thus prone to form aberrant links to other HC particularly in the oxidizing environment of the ER. Folded dimers could emerge from the unfolded dimer pool, and/or form later after folded monomers (HC/peptide/β2m) lose β2m during transport or at the cell surface. The tendency of B27.A23, HLA-B7, and mouse alleles to display the late dimerization phenotype without significant ER dimerization indicates that this pool does not have to arise from ER dimers. B pocket mutants like B27.E45M that fold rapidly and avoid ER dimerization might also avoid late dimerization if the mutation results in greater HC/peptide-β2m complex stability compared with wild type HLA-B27 or the B27.A23 mutant.

The reduced accumulation of unfolded HLA-B27 dimers in the absence of TAP (T2.B27 and 5.2.4 cells), and their prominence in the absence of tapasin (220.B27 cells) or β2m, suggests that TAP-transported peptides may contribute to their formation and/or stabilization, whereas tapasin facilitates their elimination. In support of this, it is clear from several
studies that TAP-transported peptides can stabilize and be presented by monomeric HLA-A2 and -B27 complexes in the absence of tapasin (61–64). Recent studies have shown that tapasin forms a disulfide link with ERp57 in the peptide-loading complex (9). Disruption of this link by mutating the Cys65 in tapasin prevents complete oxidation of the class I HC at the conserved Cys103/Cys164 disulfide, and results in poor peptide loading of HC/β2m heterodimers (9). Thus it is possible that the effect of tapasin on reducing the accumulation of unfolded HLA-B27 HC dimers that we observe may be via recruitment into the peptide-loading complex where they are subject to further quality control. This is consistent with the effect of tapasin on reducing the accumulation of unfolded HLA-B27 HC dimers that we observe may be via recruitment into the peptide-loading complex where they are subject to further quality control.

Both TAP and tapasin appear necessary for the recovery of W6/32-reactive folded dimers. This could reflect a specific effect on peptide loading of dimeric HC and/or an overall increase in the expression of stable monomeric HC-peptide-β2m complexes on the cell surface that are then available to dimerize. The latter explanation seems unlikely to account for the tapasin effect, because the expression of W6/32-reactive HLA-B27 HC in .220.B27 cells without tapasin is quite high, yet no folded dimers are detected. Folded HLA-B27 dimers are readily detected in T5-1 and other B-LCL with a single copy of the tapasin complex (9). This would also be consistent with the heterogeneity of the HC10-reactive dimers being a result of variable and incomplete HC oxidation. Further experiments will be necessary to test these possibilities.

It is also worth mentioning that transgenic rats expressing HLA-B27 with Ser substituted for Cys (B27.C67S) develop the spondyloarthropathy-like phenotype (spontaneous inflammatory disease) with somewhat less arthritis than controls expressing wild type HLA-B27 (21), implying that dimers per se are not required for disease. However, it should be noted that these rats have a much higher ratio of B27.C67S to human β2m transgene (3:1), whereas in the 21-3 rats the transgene ratio is 1:3:1. The transgene ratio in the B27.C67S rats is thus more than twice as high, and also considerably higher than other disease-prone HLA-B27 lines (0.83 and 1.7) (21). Therefore, the B27.C67S rats are likely to have greater expression of HC relative to human β2m. Because rodent β2m is less efficient at promoting HLA class I HC folding and expression (68), this is likely to exacerbate HLA-B27 misfolding. To the extent that Cys might normally contribute to the complete HLA-B27 misfolding phenotype, HC overexpression relative to β2m in B27.C67S rats could compensate for the loss of Cys by increasing HLA-B27 misfolding. It is therefore possible that although Cys67 is not required in this situation, it might still contribute to disease pathogenesis under more physiological conditions.

The results presented here support the idea that HLA-B27 has aberrant immunobiological characteristics related to amino acid residues that form the B pocket, but are unlikely to be related to the role of this pocket in peptide selection per se. Our data suggest that the combination of delayed HC folding and ER retention, together with an unpaired Cys that is exposed when the molecule is unfolded, may create a complete misfolding phenotype which includes aberrant disulfide bonding in the ER. Indeed, there are few alleles that fit these two criteria, and only one that also contains a Lys residue at position 70 (HLA-B73) (17), which could be important as the local chemical environment can affect the reactivity of unpaired Cys residues (15).

Acknowledgments—We thank the following investigators for kindly providing reagents or cell lines: Peter Cresswell (T2, C1R.B53, and B10.7), Tom Griffin (T2.TAP1/2), Charles Lutz (C1R.B7), Betsy Mellins (T5-1), Hidde Ploegh (HC10), Sarah Rowland-Jones (HF), Tom Spies (TAP1/2 cDNAs, C1R.B8, and .220), and Joel Taurog (B27.C67A).

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### Notes
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