Multimers That Bind to the Chaperone BiP and Disulfide-Linked Heavy Chain Oligomers HLA-B27 in Transgenic Rats Forms

Tri Minh Tran, Nimman Satuntira, Martha L. Dorris, Ekkehard May, Andrew Wang, Eiichi Furuta and Joel D. Taurog

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HLA-B27 in Transgenic Rats Forms Disulfide-Linked Heavy Chain Oligomers and Multimers That Bind to the Chaperone BiP

Tri Minh Tran, Nimman Satumtira, Martha L. Dorris, Ekkehard May, Andrew Wang, Eiichi Furuta, and Joel D. Taurog

To test the hypothesis that HLA-B27 predisposes to disease by forming disulfide-linked homodimers, we examined rats transgenic for HLA-B27, mutant Cys67Ser HLA-B27, or HLA-B7. In splenic Con A blasts from high transgene copy B27 lines that develop inflammatory disease, the anti-H chain mAb HC10 precipitated four bands of molecular mass 78–105 kDa and additional higher molecular mass material, seen by nonreducing SDS-PAGE. Upon reduction, all except one 78-kDa band resolved to 44 kDa, the size of the H chain monomer. The 78-kDa band was found to be BiP/Grp78, and the other high molecular mass material was identified as B27 H chain. Analysis of a disease-resistant low copy B27 line showed qualitatively similar high molecular mass bands that were less abundant relative to H chain monomer. Disease-prone rats with a Cys67Ser B27 mutant showed B27 H chain bands at 95 and 115 kDa and a BiP band at 78 kDa, whereas only scant high molecular mass bands were found in cells from control HLA-B7 rats. 125I-surface labeled B27 oligomers were immunoprecipitated with HC10, but not with a mAb to folded B27-β2-microglobulin-peptide complexes. Immunoprecipitation of BiP with anti-BiP Abs coprecipitated B27 H chain multimers. Folding and maturation of B27 were slow compared with B7. These data indicate that disulfide-linked intracellular H chain complexes are more prone to form and bind BiP in disease-prone wild-type B27 and B27-C67S rats than in disease-resistant HLA-B7 rats. The data support the hypothesis that accumulation of misfolded B27 participates in the pathogenesis of B27-associated disease. The Journal of Immunology, 2004, 172: 5110–5119.

HLA-B27 refers to a family of closely related alleles of the human MHC class Ia HLA-B locus. B27 is of particular interest because it is very highly associated with the chronic inflammatory disorder ankylosing spondylitis and related rheumatic conditions known as spondylarthritides or spondyloarthropathies. In most populations, B27 is found in ~90% of patients with ankylosing spondylitis, compared with 5–8% of the general population (relative risk ~100). It can be concluded that the B27 molecule itself is involved in disease pathogenesis, based on strong evidence from genetic epidemiology and on findings in B27 transgenic rodents (reviewed in Ref.1). The molecular basis for the propensity of the B27 molecule to induce disease is not known, but it presumably derives from one or more unique features of B27 biology.

The B27 subtypes that are known to be associated with disease share a common B pocket in the peptide binding groove, containing characteristic residues His9, Thr24, Glu45, and Cys67, with adjacent residues Ala69, Lys70, and Ala71 (2). This deep, negatively charged pocket shows a strong preference for binding the arginine side chain, explaining the preference of the B27 binding groove for peptides with Arg at the second position, as suggested by the crystal structure of B27 and confirmed by peptide isolation and sequencing (3, 4). In addition, B27 is among the most negatively charged of HLA class I H chains (5), and the overall preference is for positively charged peptides. The Cys67 residue is shared with few other MHC-I alleles, and the combination of Cys67 and Lys70 (which promotes sulfhydryl reactivity) is almost unique to B27.

Despite 30 years of investigation, the mechanism by which HLA-B27 predisposes to inflammatory disease is not yet understood. Although the participation of the peptide-binding specificity of HLA-B27 in the pathogenesis of spondylarthritides has by no means been excluded, efforts to identify a disease-associated B27-restricted CD8 T cell response have met with only occasional success (6). Investigation of the spontaneous spondyloarthropathy-like inflammatory disease that develops in HLA-B27 transgenic rats (7, 8) has not found a role for conventional CD8 T cells in this disease (9). In contrast, B27 has been found to possess several unusual features compared with other MHC-Ia molecules and it has been suggested that the association of B27 with disease is related to one or more of these features.

MHC class I molecules are assembled in the endoplasmic reticulum (ER). The nascent H chain becomes glycosylated and associates with the chaperone calnexin. Subsequently, the calnexin-associated H chain forms a heterodimer with β2-microglobulin (β2m), the calnexin dissociates, and the H chain-β2m heterodimer binds the multisubunit peptide-loading complex consisting of the soluble lectin chaperone calreticulin, the thiol oxidoreductase

Abbreviations used in this paper: ER, endoplasmic reticulum; β2m, β2-microglobulin, DPBS, Dulbecco’s PBS; hβ2m, human β2m; NEM, N-ethylmaleimide.
Erp57, the transmembrane glycoprotein tapasin, and the heterodimer ATP-dependent transporter TAP (10). Peptides transported by TAP into the ER from the cytoplasm are trimmed at the N terminus and become bound within peptide-binding grooves of compatible MHC-I H chain alleles (11). The folded H chain-β2m-peptide trimer then dissociates from the loading complex and proceeds to the cell surface via the secretory pathway through the Golgi apparatus. Some aspects of this pathway are shared in common with other ER-assembled glycoproteins, whereas other features, such as the association with TAP and tapasin, are more specific for MHC class I assembly (12).

In human cell lines, B27 assembly in the ER is usually inefficient, compared with other HLA class I molecules, with a high rate of misfolding and translocation of free H chain into the cytosol, where it is degraded (13, 14). Unconventional recognition of B27 by CD4+ T cells has been reported (15). As a MHC-Ia molecule, B27 not only binds peptides but also interacts at different sites on the molecule with β2m, TCR, CD8α, and molecules such as KIR3DL1 expressed on NK cells (16, 17). B27 seems less dependent on tapasin for peptide loading than other HLA alleles (18). It has also been reported that disulfide-linked B27 H chain homodimers form within the ER and may also be expressed on the cell surface (14, 19, 20), and substitution of alanine or serine for the free Cys67 residue inhibited the formation of these dimers. It has been speculated that B27 homodimers play a significant role in the pathogenesis of B27-associated disease (17, 20). An unusual form of surface-expressed B27 that is loaded with unconventional long peptides and lacks β2m has been described (21).

As noted above, rats transgenic for high gene copy numbers of HLA-B27 and human β2m (hβ2m) develop a multisystem inflammatory disease that serves as a model for human B27-associated disease (7, 8). In these studies, we examined the biosynthesis of B27 and the formation of H chain homodimers in spleen cells from rats transgenic for B27, for a mutant B27 gene encoding Ser67, or for HLA-B7. The results are consistent with a correlation between B27 and the formation of H chain homodimers in spleen cells from rats transgenic for high gene copy numbers of HLA-B27 and human β2m (hβ2m) develop a multisystem inflammatory disease, intracellular H chain disulfide-linked multimer formation, and binding the chaperone BiP. The Cys67 is not essential for any of these processes.

**Materials and Methods**

**Rats**

The transgenic lines listed in this study, summarized in Table I, have all been previously described (7, 8, 22). All lines have been backcrossed to the Lewis (LEW) background for at least 20 generations. All 133-1 and 120-4 rats studied were homozygous for the transgene locus. Nontransgenic LEW rats were used as controls. The 133-1 line was confirmed to carry the Cys67Ser B27 mutation by sequencing of the B27 transgene.

**Antibodies**

B9.12.1 (mAb mouse IgG2a) recognizes a conformation-dependent epitope on HLA class I molecules that requires β2m and peptide binding (23). We chose B9.12.1 instead of W6/32, an anti-HLA class I mAb widely used in studies of human cells, because B9.12.1, unlike W6/32, neither binds rodent MHC class I in the presence of hβ2m or bovine β2m, nor fails to bind B27 in complex with rodent β2m (Refs. 24 and 25, and our unpublished observations). HC10 (mAb mouse IgG2a) recognizes an epitope in the α1 domain of unfolded HLA-B and -C H chains that are not assembled with β2m (5, 26). 3B10.7 is a rat IgM mAb with broad specificity for human class I H chain alleles and is not conformation dependent (27). Anti-BiP (anti-Grp78) rabbit polyclonal Ab was purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada).

**Metabolic labeling**

Single-cell suspensions of Ficoll-Hypaque-purified rat spleen cells were prepared as previously described (28). A total of 10^7 spleen cells were cultured at 2 × 10^6 cells/ml overnight at 37°C in complete RPMI 1640 medium supplemented with 10% FCS (R-10) and Con A (2.5 μg/ml). Then, cells were preincubated for 1 h at 37°C in 500 μl of R-10 medium lacking Met and Cys, followed by labeling with 0.25 mCi of[35S]Met/Cys (Trans-S-Label; ICN Pharmaceuticals, Costa Mesa, CA) for 4 h. Protein synthesis was stopped by the addition of 5 vol of ice-cold Dulbecco's phosphate buffer saline (DBPS), and cells were harvested by centrifugation, then washed again. Cells were then incubated for 10 min at 4°C with 20 mM N-ethylmaleimide (NEM) to block free sulphydryl groups and protect native disulfide bonds during cell lysis (29), after which the cells were washed, pelleted, and frozen at −80°C. For analysis, frozen cell pellets were lysed at 4°C for 1 h in 500 μl of lysis buffer (LB), consisting of 20 mM Tris (pH 7.8), 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5 mM iodoacetamide, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.04% sodium azide, and 1 mM PMSF (Ref. 14 with modifications). Nuclei and cell debris were then pelleted at 10,000 × g for 5 min at 4°C, and supernatants were used for immunoprecipitation.

**Pulse-chase labeling**

Cells (5 × 10^6 per time point) were preincubated in Met/Cys-deficient R-10 medium (1 × 10^6 cells/ml) for 1 h at 37°C, pulse-labeled with 0.25 mCi of[35S]Met/Cys (ICN Pharmaceuticals) for 5 min, then chased in a 10-fold excess of medium containing 2 mM Met/Cys. At the end of each time point, cells were handled as described under Metabolic labeling.

**Endoglycosidase H digestion**

Proteins were digested with Endo H following the manufacturer’s instructions (NEB, Beverly, MA). In brief, proteins were eluted from immunoprecipitates by boiling for 10 min in 15 μl of 2X denaturing buffer (1% SDS, 2% 2-ME). The supernatant was then collected and treated with 500 U of Endo H in 50 mM sodium citrate (pH 5.5) overnight at 37°C.

**Cell surface iodination**

Iodination of B27 molecules on the cell surface was performed in IODOGEN precoated iodination tubes (Pierce, Rockford, IL), following the manufacturer’s instructions. In brief, the spleen cell suspension (1 × 10^7 cells/ml DBPS) was added to an IODOGEN precoated iodination tube, followed by incubation with 500 μCi Na125I (carrier free) (NEN, Boston, MA) for 15 min at room temperature. The reaction was stopped by transferring the cells to another tube and adding tyrosine (1 μM) to bind the residual 125I. The cells were then washed twice with ice-cold DBPS supplemented with 5 mM KI and used for immunoprecipitation. Some cell samples were treated with NEM or iodoacetamide, either before or after iodination, as described in Results.

<table>
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<th>Linea</th>
<th>HLA H Chain Transgene</th>
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<th>hβ2m Transgene Copy No.b</th>
<th>Disease</th>
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<td>90</td>
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</tr>
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<td>21–4L</td>
<td>B*2705</td>
<td>6</td>
<td>6</td>
<td>No</td>
</tr>
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<td>L33–3</td>
<td>B*2705</td>
<td>55</td>
<td>66</td>
<td>Yes</td>
</tr>
<tr>
<td>133–1</td>
<td>B*2705 Cys67Ser</td>
<td>12</td>
<td>4</td>
<td>Only in homozygotes^c</td>
</tr>
<tr>
<td>120–4a</td>
<td>B*0702</td>
<td>26</td>
<td>5</td>
<td>No</td>
</tr>
</tbody>
</table>

^a All lines are on the inbred LEW background.

^b Gene copy number in the homozygous transgene locus.

^c All rats used from these lines were homozygous for the transgene locus.

^d A cohort of 50 133-1 homozygotes was examined for onset of arthritis, which developed in 8 of 27 males at a mean age of 234 ± 56 days (median 260 days), and in 4 of 23 females at a mean age of 217 ± 45 days (median 209 days). In contrast, wild-type 21–4H and L33-3 males show a median age of arthritis onset of 120 days (9). The 133-1 rats developed colitis similar to the wild type (data not shown).

Table I. Transgenic rat lines used in this study
**Immunoprecipitations**

Labeled or unlabeled cells were lysed in Triton X-100, as described above. Immunoprecipitations were performed after preclearing samples with washed formalin-fixed *Staphylococcus aureus* and protein A-Sepharose (Sigma-Aldrich, St. Louis, MO), using purified mAbs at a final concentration of 30 μg/ml for 1 h at 4°C. Protein A-Sepharose was then added (100 μl of a 125 mg/ml suspension in lysis buffer) for 1 h at 4°C. Pellets were washed sequentially with PLB containing 0.1% SDS and 1% BSA, LB containing 1% Triton X-100, a 10-fold dilution of LB supplemented to 80 mM NaCl, and LB alone, and then stored at −20°C until electrophoresis. All buffers in all steps of the immunoprecipitations contained 5 mM iodoacetamide to block free sulfhydryl groups.

**Gel electrophoresis and autoradiography**

Samples were analyzed by SDS-PAGE on 10.5% acrylamide gels (30). For reducing and nonreducing SDS-PAGE, samples were boiled for 5 min in an equal volume of 2× sample buffer with or without DTT (200 mM), respectively, before electrophoresis. For autoradiography, gels were fixed in 30% methanol/10% acetic acid for 30 min followed by treatment with Enlightening solution (NEN) for 30 min, and then dried. Dried gels were stored in deionized H2 O. Visualized protein spots were analyzed and sequenced by the University of Texas Southwestern Protein Sequencing Core Facility, using purified streptavidin (Antibodies, Inc., Davis, CA) for 35 min, then bound Ab (Southern Biotechnology Associates, Birmingham, AL) or HRP-conjugated streptavidin (Antibodies, Inc., Davis, CA) for 35 min, then bound Ab was visualized using ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

**Image quantitation**

Gel images were quantitated by scanning on an Alpha Imager 2200 (Alpha Innotech, San Leandro, CA).

**Results**

**HC10-reactive H chain oligomers are abundantly generated in cells from HLA-B27 and HLA-B27-C67S rats but not HLA-B7 transgenic rats**

To investigate whether B27 H chain multimers form during B27 biosynthesis in B27 transgenic rats, spleen cells from the disease-prone high copy B27 transgenic lines L.33-3 and 21-4H, the non-disease-prone low copy B27 transgenic line 21-4L, the B27 line 133-1 carrying a C67S mutation, and the HLA-B7 line 120-4 were cultured with Con A, metabolically labeled, then lysed, and the immunoprecipitates with mAbs HC10 or B9.12.1 were separated by SDS-PAGE under nonreducing and reducing conditions. As shown in the autoradiograms in Fig. 1, the two wild-type B27 lines, L.33-3 and 21-4H, showed identical patterns with five bands appearing between 68 and 105 kDa in the HC10 immunoprecipitate under nonreducing conditions, along with other higher molecular mass material (Fig. 1A, lanes 3 and 4, bands a–e). The pattern...
for the 21-4L line was qualitatively similar, but quantitatively much less. The mutant B27 line 133-1 showed four bands between 68 and 115 kDa, two bands apparently shared with wild-type B27 (Fig. 1A, bands d and e) and two distinct bands (Fig. 1A, bands a’ and b’). In contrast, the HLA-B7 line showed no bands in this region, although very high molecular mass material was evident at the top of the gel. Under reducing conditions, band d at 78 kDa remained in all four B27 lysates, whereas most of the other high molecular mass material resolved to the H chain monomer band or smaller bands in all five lines (Fig. 1B). Unlike HC10, the mAb B9.12.1, which binds only fully folded HLA class I molecules, did not precipitate any bands larger than the H chain monomer. These results suggested that during biosynthesis, the B27 H chain, but not the B7 H chain, forms disulfide-linked oligomers, either with itself or with other ligands, or both. To examine the composition of the oligomer bands, Con A blasts from 21-4H, 133-1, 120-4, and control (nontransgenic) spleen cells were either metabolically labeled or not, then subjected to lysis, immunoprecipitation, and SDS-PAGE as described above. The gels from the nonlabeled material were blotted onto nitrocellulose and probed with the anti-HLA class I H chain mAb 3B10.7, whereas the gels from the labeled material were subjected to autoradiography. As shown in the non-reducing gel of material immunoprecipitated with HC10 in Fig. 2A, in the 21-4H lysate, bands apparently corresponding to bands a, b, and c in the autoradiogram were reactive in the immunoblot with 3B10.7. In the 133-1 lysate, bands apparently corresponding to bands a’ and b’ were reactive with 3B10.7, along with a third less distinct band just below b’. One faint 89-kDa band was also seen in the 120-4 lysate. In the reducing gels, a band at 78 kDa was found in the 21-4H and 133-1 lysates, corresponding to band d in the autoradiogram (Fig. 2C) but not the immunoblot (Fig. 2D). The immunoblot data of material precipitated by HC10 indicate that bands a, b, c, a’, and b’ likely contain B27 H chain, whereas band d represents a different molecular species that is not affected by reduction.

Immunoblotting with 3B10.7 of B9.12.1 immunoprecipitates consistently showed the presence of three bands between 90 and 113 kDa in the 21-4H spleen cells and a single band at 97 kDa in the 120-4 spleen cells, but no high molecular mass bands in the 133-1 spleen cells (Fig. 2F). Because these bands are not apparent after metabolic labeling up to 4 h (Figs. 1C, 2E, and data not shown), and because they are not evident in surface-labeled material (see below), they apparently represent folded H chain that exists in an intracellular compartment either as a homodimer or in complex with another protein. The absence of any such bands in the 133-1 cells and the presence of one in the 120-4 cells together suggest that these bands are not related to the disease process.

All high molecular mass bands except band d correspond to B27 H chain

To further characterize the composition of the high molecular mass bands, HC10 immunoprecipitates were analyzed in two-dimensional nonreducing/reducing gels. Fig. 3A shows an autoradiogram from a metabolically labeled 21-4H Con A blast spleen cell lysate immunoprecipitated with HC10. In reducing gels, bands a, b, and c, resolved to the size of the H chain monomer, whereas the 78-kDa band d remained on the diagonal. Much of the higher molecular mass material also resolved to one band the size of the H chain monomer, suggesting that the B27 H chain forms a variety of complex disulfide-linked multimers. The absence of additional spots in the reducing gel suggests that the B27 H chain bands at ~90–110 kDa are homodimers, or else represent B27 in complex with one or more molecules that do not label with [35S]Met/Cys under these conditions. Immunoblotting with 3B10.7 of a corresponding two-dimensional gel (Fig. 3B) confirmed that the three discrete high molecular mass bands and accompanying higher and lower molecular mass material contain B27 H chain, whereas the spot corresponding to band d does not.

Similar results were obtained in nonreducing/reducing gel analysis of the 133-1 mutant B27 line. The two bands a’ and b’ resolved to one band the size of the H chain monomer in both metabolically labeled (Fig. 3C) and immunoblotted (Fig. 3D) HC10 immunoprecipitates, and both reacted with 3B10.7, whereas band d did not.

B27 H chain reactive with HC10, but not B9.12.1, is present on the cell surface

To explore whether B27 oligomers are present on the cell surface, Con A blasts were surface labeled with 125I. Because the iodination reaction involves vigorous oxidation that might artifically
create or alter disulfide bonds, we compared the effect of pretreatment of the cells with NEM and/or iodoacetamide before iodination with iodination in the absence of this treatment. As shown in the nonreducing gels in Fig. 4A, when L.33-3 Con A blasts were either pretreated with NEM before iodination or iodinated in the presence of iodoacetamide, immunoprecipitation with HC10 yielded a moderate amount of somewhat diffusely distributed material of 66–105 kDa (lanes 1 and 2), whereas cells that were treated with NEM only after iodination, or not treated at all (lanes 3 and 4, respectively), showed more prominent bands at ~96 kDa, abundant higher molecular mass material, and much weaker monomer bands. Even more striking was the result with B9.12.1, in which the NEM-pretreated cells or cells iodinated in the presence of iodoacetamide showed no high molecular mass bands (lanes 1 and 2), whereas the cells not subjected to either of these treatments before or during iodination showed prominent bands at ~90–105 kDa as well as abundant higher molecular mass material. These results suggest that there is normally no multimer of folded B27 on the cell surface, and that unfolded B27 H chain reactive with HC10 exists on the cell surface predominantly as monomer, but also within a spectrum of multimer species.

Iodination of cells pretreated with NEM was repeated using Con A-treated spleen cells from the 120-4, 133-1, 21-4L, and 21-4H lines. As shown in Fig. 5A, a series of bands was seen between 66 and 120 kDa in the cells from all four lines, which were more prominent in the two wild-type B27 lines (21-4H and 21-4L). These bands were nearly abolished by reduction with DTT (Fig. 5C). Again, no high molecular mass bands were seen in the material immunoprecipitated with B9.12.1 (Fig. 5, B and D). These results confirm that B27 H chain oligomer and multimers are present on spleen cell surfaces in the B27 transgenic rats, but very little if any that is folded in a native conformation.

All of the 80–110 kDa bands contain B27 except band d, which is BIP

To further characterize the high molecular mass bands, HC10 reactive material was immunoaffinity purified from a lysate of pooled 21-4H and L.33-3 spleen cells, and then analyzed on a preparative nonreducing/reducing two-dimensional gel that was stained with colloidal Coomassie blue (Fig. 6). In the nonreducing gel, band d was most abundant, followed by band a, with bands b and c barely visible. In the reducing gel, all four bands were evident. Sequencing by mass spectrometry of tryptic digests of material eluted from the bands yielded results shown in Table II. All of the sequenced bands showed HLA-B27 to be the predominant sequence, major vault protein (32), comigrated with band d from the transgenic lines and was also identified at the same gel position in the nontransgenic control immunoprecipitate. Similar results were obtained with cells from the 133-1 mutant line, where bands
and precipitates (\textit{A} and \textit{C}) show a band at ~79 kDa. A 38-kDa band is present in all of the HC10 precipitates (\textit{A} and \textit{C}), but in the nonreducing gel it is much more prominent in 120-4 and 133-1 lanes than in the two wild-type B27 lanes. The nonreduced B9.12.1 precipitate shows very little high molecular mass material below 203 kDa for any of the lines, similar to the findings for L.33-3 in Fig. 4. NTG, Nontransgenic.

$\alpha'$ and $\beta'$ were confirmed to contain predominantly B27, and $\beta$ was again found to contain predominantly BiP. Taken together, the results thus far indicate that B27 H chains form oligomers during biosynthesis, and that at least some of these oligomers contain the chaperone BiP.

\textit{BiP is abundantly associated with the high molecular mass forms of B27 H chain}

To help estimate the extent that BiP is bound to B27 H chain, immunoprecipitates from lysates of L.33-3 or nontransgenic spleen cells that were immunoprecipitated with HC10, 3B10.7, or B9.12.1 conjugated to Sepharose were analyzed by single dimension nonreducing SDS-PAGE and colloidal Coomassie blue staining. As shown in Fig. 7A, a prominent band corresponding to BiP was brought down by HC10, associated with B27 H chain monomer and higher molecular mass material. Similar results were obtained with 3B10.7 (data not shown). No BiP was evident in the nontransgenic lysate or in the B9.12.1 precipitates. Next, spleen cells from L.33-3 or nontransgenic rats were metabolically labeled and lysates were immunoprecipitated with an anti-BiP Ab or with HC10 and analyzed by SDS-PAGE under nonreducing or reducing conditions. As shown in Fig. 7B, the anti-BiP Ab coprecipitated BiP and B27 H chain, the latter apparently existing predominantly as disulfide-linked oligomers that resolved to the size of the monomer under reducing conditions. The anti-BiP Ab precipitated more BiP from the L.33-3 cells than from the nontransgenic cells. As shown in Fig. 7C, immunoblotting with 3B10.7 of parallel nonlabeled anti-BiP immunoprecipitates separated by SDS-PAGE confirmed that under nonreducing conditions, predominantly high molecular mass B27 H chain was associated with BiP, with little or no B27 H chain monomer.

A quantitative estimate of BiP binding was conducted by analyzing Coomassie blue-stained nonreducing gel of material immunoaffinity purified with HC10, as described above for Fig. 6. The intensity of staining for BiP was calculated relative to the starting number of cells, and then normalized to that obtained from the pool of 21-4H and L.33-3 cells. As shown in Fig. 8A, the amount of BiP per cell copurified with HLA H chain was at least 6- to 9-fold higher in the 21-4H/L.33 and 133-1 cells than in 120-4 cells. Similar results were found by metabolic labeling, for example in the BiP and H chain bands shown in the reducing gel in Fig. 1B, where BiP is virtually absent from the 120-4 lane. To further quantify the relationship of BiP and H chain, lysates from metabolically labeled Con A blasts were quantitatively immunoprecipitated with anti-BiP and analyzed by reducing SDS-PAGE. As shown in Fig. 8B, after subtracting the background, the three B27 lines

![Figure 5](image1)

![Figure 6](image2)

**Table II. Proteins identified from gels in Fig. 6**

<table>
<thead>
<tr>
<th>Rat Line(s)</th>
<th>Gel</th>
<th>Band/Spot</th>
<th>Protein(s) Identified</th>
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<td>$\alpha'$</td>
<td>HLA-B27</td>
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<td></td>
<td>$\beta'$</td>
<td>Major vault protein (32)</td>
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<tr>
<td></td>
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<td>$\delta$</td>
<td>BiP</td>
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</table>
B27 was purified from 9 × 10⁷ spleen cells from the 21-4H, 21-4L, 133-1, and 120-4 lines were used to assess the time course of maturation to folded and fully glycosylated HLA-B27 in the ER. Con A blastlets from the 21-4H line showed a slow assembly of the HLA transgene products in rats, consistent with the previously reported data.

The formation of B27 multimers and complexes with BiP is consistent with previous studies (34). BiP coprecipitates with B27 H chain multimers. A prominent band at 78 kDa (arrowhead) is precipitated by HC10 along with B27 H chain monomer (arrow) and diffuse high molecular mass material (bracket), but not with B9.12.1. B. Spleen cells from L.33-3 or nontransgenic rats were metabolically labeled for 4 h. Immunoprecipitates from the lysates were made with anti-BiP/Grp78 or HC10, followed by separation on SDS-PAGE under nonreducing or reducing conditions. B27 H chain monomer appears in the anti-BiP immunoprecipitate only under reducing conditions, suggesting that under nonreducing conditions BiP only associates with B27 H chain multimers. However, a faint monomer band became evident upon longer exposure (not shown). Each lane represents 2.5 × 10⁶ cell equivalents. C. Spleen cells from L.33-3 or nontransgenic rats were lysed and immunoprecipitated from the lysates were made with anti-BiP/Grp78, resolved on SDS-PAGE under nonreducing or reducing conditions, blotted onto nitrocellulose, and probed with 3B10.7, followed by detection with goat anti-rat IgG Ab-HRP conjugate. Under nonreducing conditions, specific staining is only seen in the region of 90–120 kDa (bracket). Under reducing conditions, specific staining is only seen at the size of the B27 H chain monomer (arrow). NTG, Nontransgenic; NR, nonreducing; R, reducing.

**FIGURE 7.** BiP coprecipitates with B27 H chain multimers. A, HLA-B27 was purified from 9 × 10⁷ spleen cells from L.33-3 using HC10- or B9.12.1-CNBr Sepharose, followed by nonreducing SDS-PAGE and staining with colloidal Coomassie blue. A prominent band at 78 kDa (arrowhead) is precipitated by HC10 along with B27 H chain monomer (arrow) and diffuse high molecular mass material (bracket), but not with B9.12.1. B, Spleen cells from L.33-3 or nontransgenic rats were metabolically labeled for 4 h. Immunoprecipitates from the lysates were made with anti-BiP/Grp78 or HC10, followed by separation on SDS-PAGE under nonreducing or reducing conditions. B27 H chain monomer appears in the anti-BiP immunoprecipitate only under reducing conditions, suggesting that under nonreducing conditions BiP only associates with B27 H chain multimers. However, a faint monomer band became evident upon longer exposure (not shown). Each lane represents 2.5 × 10⁶ cell equivalents. C. Spleen cells from L.33-3 or nontransgenic rats were lysed and immunoprecipitated from the lysates were made with anti-BiP/Grp78, resolved on SDS-PAGE under nonreducing or reducing conditions, blotted onto nitrocellulose, and probed with 3B10.7, followed by detection with goat anti-rat IgG Ab-HRP conjugate. Under nonreducing conditions, specific staining is only seen in the region of 90–120 kDa (bracket). Under reducing conditions, specific staining is only seen at the size of the B27 H chain monomer (arrow). NTG, Nontransgenic; NR, nonreducing; R, reducing.

**FIGURE 8.** Significantly more BiP is associated with B27 H chain in the disease-prone B27 lines than with B7 H chain in the 120-4 line. A, Lysates of spleen cells from nontransgenic (5.9 × 10⁹), 120-4 (5.9 × 10⁹), pooled 21-4H/L.33-3 (2.7 × 10⁹), or 133-1 (5.3 × 10⁹) rats were passed through a 0.5-ml column of CNBr-activated Sepharose CL-4B coupled to 3 mg of HC10, as described in Materials and Methods. Half of the eluates were resolved by SDS-PAGE and then stained with colloidal Coomassie blue. The bands shown here are the BiP bands from this gel. The gel was photographed and scanned for image density, and the density per cell was calculated and normalized to the 21-4H/L.33-3-pooled cells. The other half of the eluates was subjected to two-dimensional analysis before staining, as shown in Fig. 6. The 133-1 BiP band shown here is from the same eluate as the one analyzed in Fig. 6C and corresponds to band d on the nonreducing gel in Fig. 6C. The materials used in Fig. 6, A and B, were from a separate experiment. B, Con A blast spleen cells from the indicated rat lines were labeled 4 h with [³⁵S]Met/Cys and cell lysates were subjected to exhaustive immunoprecipitation (three times) with anti-BiP and the respectively pooled precipitates were analyzed by SDS-PAGE under reducing conditions. Each lane represents material equivalent to 2.5 × 10⁶ cells. Bands corresponding to BiP and H chain (arrows) were quantitated. The lower band of the doublet at molecular mass ~44,000 Da is presumed to be the site of the B27 H chain, because the upper band of the doublet is invariant between transgenic and nontransgenic lines. The H chain/BiP ratio was calculated after subtracting the background nontransgenic lower band density from the lower band density of the other four lines. NTG, Nontransgenic.

**Discussion**

Spleen cells from rats transgenic for HLA-B27 were found to generate B27 H chain homodimers, consistent with previous data from human cell lines (14, 20). The homodimers appeared during biosynthesis, were readily detected with the mAbs HC10 and 3B10.7, and were shown by nonreducing/reducing two-dimensional gels and by direct protein sequencing to not contain significant amounts of other molecular species. Higher order multimers containing the B27 H chain were also evident, and consistent resolution of all of the B27 H chain bands to the monomer size upon reduction with DTT suggests that they are disulfide linked. The B27 H chain also forms a complex with the ER chaperone BiP, as previously suggested by others (14, 35). HC10-reactive B27 dimers were found in the wild-type B27 and B27-C67S transgenic rats, both of which are disease prone, but not in HLA-B7 control rats, which remain healthy. Surface labeling with [¹²⁵I] under conditions designed to

**HLA-B27 is slow to assemble and slow to exit the ER in transgenic rat spleen cells**

The formation of B27 multimers and complexes with BiP is consistent with the previously reported finding that B27 is slowly and inefficiently assembled in cell lines (13, 33). To test the speed of assembly of the HLA transgene products in rats, Con A blast spleen cells from the 21-4H, 21-4L, 133-1, and 120-4 lines were subjected to two types of pulse-chase analysis with [³⁵S]Met/Cys. In the first type, aliquots of labeled cells were taken at intervals, divided in half, and exhaustively immunoprecipitated with either HC10 or B9.12.1. As shown in Fig. 9, A and B, the progression from unfolded H chain recognized by HC10 to folded H chain recognized by B9.12.1 was slowest in the 21-4H and 133-1 cells, fastest in the 120-4, and intermediate in the 21-4L cells. In the second type of analysis, samples taken at intervals were immunoprecipitated with B9.12.1 and treated with endoglycosidase H to assess the time course of maturation to folded and fully glycosylated complexes within the medial Golgi (34). As shown in Fig 9, C and D, HLA-B7 was rapidly assembled, whereas all three B27 lines, i.e., the high copy disease-prone 21-4H line, the low copy non-disease-prone 21-4L line, and the mutant B27 133-1 line, showed nearly identical rates of assembly that were considerably slower than that of HLA-B7. These results indicate that the assembly of HLA-B27 in the ER is slower than that of HLA-B7 throughout its biosynthesis, and that the initial folding of the free H chain is more inefficient in the high copy and mutant B27 lines than in the low copy B27 line.
B27 molecules is particularly susceptible to dimer formation. If so, it may be that this pool of unloaded or suboptimally loaded C67S mutant. It has recently been suggested that unloaded or sub-

from rat lines expressing wild-type B27 and B7, but not the B27-

these dimers within the cells, and to explain how they form in cells

lines (14). Further studies will be needed to identify the location of

the indicated rat lines were pulsed for 5 min with [35S]Met/Cys and chased for the indicated time, and lysates were divided into two equal parts and subjected to three sequential immunoprecipitations with either HC10 or B9.12.1. The respective immunoprecipitates were pooled and analyzed by reducing SDS-PAGE and autoradiography. The H chain bands are shown. Each lane represents material equivalent to 2.5 x 10^6 cells. B, The bands in A were quantitated and the percentage of HC10 band intensity to the total HC10 + B9.12.1 was calculated and plotted. The 21-4H and 133-1 lines show the slowest rate of folding. The 21-4L folding rate is intermediate, and the 120-4 rate is fastest. C, Con A blast spleen cells from the indicated rat lines were pulsed for 5 min with [35S]Met/Cys and chased for the indicated time, and lysates were subjected to immunoprecipitation with B9.12.1. The precipitates were treated with Endo H as described in Materials and Methods and then analyzed by SDS-PAGE and autoradiography. Each lane represents material equivalent to 2.5 x 10^6 cells. D, The bands in C were quantitated and the disappearance of the Endo H-sensitive lower band was calculated. The rate of maturation of folded B7 molecules in 120-4 is approximately twice the rate of maturation of folded B27 in the other three rat lines.

The chaperone BiP was abundantly immunoprecipitated from B27 transgenic cell lysates by HC10, and conversely, B27 H chain was immunoprecipitated from B27 transgenic cell lysates by anti-

BiP. In contrast, little if any HLA-B7 H chain was bound to BiP. The results under nonreducing conditions suggest that the form of B27 H chain bound by BiP is predominantly higher multimers, but additional experiments will be needed to better characterize the B27 H chain-BiP complexes quantitatively and qualitatively. BiP is thought to stabilize nascent polypeptides through interactions with hydrophobic patches (37), thereby mediating folding, preventing aggregation and premature degradation, and suppressing formation of nonnative disulfide bonds (12, 38).

More recently, BiP has been suggested to be the master regulator of the ER stress response, the concert of signaling pathways that alters gene transcription in response to an accumulation of unfolded or misfolded proteins in the ER (39), because BiP regulates the activation of at least three transducers of ER stress, activating transcription factor-6, IRE-1, and PKR-like ER kinase (40–42). All three of these proteins have sensor domains within the ER lumen and effector domains within the cytosol, and all share binding to BiP and release from BiP as a common mechanism for regulation. In unstressed cells, BiP acts as a repressor by binding to the ER luminal domains of these proteins. In stressed cells, BiP binds to accumulating unfolded or misfolded proteins, releasing these three transducers and thus allowing activation of downstream nuclear events (40, 42).

It has been postulated (43) that B27 H chain may be unusually susceptible to triggering of the ER unfolded protein response because of its propensity to misfold. Included in this hypothesis was the proposal that accumulation of misfolded B27 may activate a proposed related pathway termed the ER overload response (44), with subsequent activation of N费αkB and its inflammatory pathways (45). Part of the basis for the formulation of this hypothesis was the observation that inflammatory disease only occurs in the B27 transgenic rat lines with high gene copy number and supraphysiologic expression of B27 (7, 8, 22), and that arthritis has been observed in mice lacking β_m, whether in the presence or absence of B27 (46, 47). The data reported in this study, that in B27 transgenic rat spleen cells B27 assembles inefficiently, forms H chain multimers, and binds extensively to BiP, support a role for B27 misfolding in inducing ER stress. Although B27 dimer bands were also observed in spleen cells from rats of the B27 transgenic line 21-4L, which has a lower transgene copy number and remains completely healthy, these dimer bands were much less abundant than in the high copy lines, both in absolute amount and in proportion to the amount of H chain monomer (Fig. 2). Moreover, the rate of H chain folding, as measured by progression from the unfolded HC10-precipitable form to the folded B9.12.1-precipitable form, was highest in the 120-4 cells, but higher in the 21-4L cells than in the 21-4H cells (Fig. 9, A and B). This suggests that the rate of folding is dependent both on the H chain allele itself and on its abundance. High levels of H chain production evidently overwhelm a rate-limiting component of the folding or disposal mechanisms, with consequent accumulation of unfolded H chain. We have previously observed a threshold effect of the level of B27 gene copy number and expression on disease susceptibility in different rat lines (8, 22), and this effect is consistent with a threshold effect of B27 H chain accumulation in the ER on the triggering of ER-to-nucleus signaling events.

In the original study identifying MHC class I binding to BiP (35), it was noted that binding to BiP and binding to calnexin were mutually exclusive, and it was postulated that the binding to BiP might represent a disposal pathway. A recent study of the pancreatic isofrom of human β-secretase, BACE457, suggests that for
this glycoprotein, which fails to fold properly, binding to BiP is indeed related to disposal, but the binding to calnexin and BiP is sequential, not mutually exclusive (48). Moreover, like HLA-B27, BACE457 was found to form disulfide-linked complexes in the ER, and this was shown to be related to binding to the oxidoreductase chaperone protein disulfide isomerase. The extent that the handling by ER chaperones of overproduced B27 H chain is similar to that found for BACE457 remains to be determined. The results of the nonreducing/reducing two-dimensional gels suggest that no additional proteins were bound to the B27 H chain in the Triton X-100-solubilized lysates. On the other hand, the formation of disulfide-linked multimers in the ER implies an interaction with a resident oxidoreductase.

The C67S mutant spleen cells showed the presence of disulfide-linked homodimers, with two B27 bands at 115 and 92 kDa, compared with the three wild-type B27 bands at 105, 94, and 82 kDa. Thus, unlike previous reports of data with transfected human or rat cell lines or recombinant-expressed proteins (14, 19, 20, 33), replacement of the Cys67 residue in B27 does not abrogate dimer formation in the transgenic rats. In addition to the polymorphic cysteine at position 67, the B27 H chain has conserved cysteine residues at positions 101, 164, 259, and 308 (49, 50). As noted above, pretreatment of cells with the membrane-permeable alkylating agent NEM, which irreversibly inactivates sulfhydryl groups and protects native disulfide bonds during cell lysis (29, 51), dramatically diminished the abundance and complexity of B27 H chain multimers, compared with cells that were not pretreated with NEM but were lysed in buffer containing iodoacetamide (Fig. 5 and data not shown). We did not systematically examine conditions to optimize use of this reagent, and thus the multimer bands seen experimentally should be considered as representing a maximum estimate of the multimers formed physiologically, because it is clear that cell lysates are highly vulnerable to disulfide bond formation in the absence of NEM. However, the bands in the H chain dimer size range that we observed were consistent from experiment to experiment and correspond well with those described by others, and hence are very likely to represent accurately the status of B27 within the intact cell.

The C67S mutant B27 transgene develop disease with a somewhat milder phenotype than the rats with wild-type B27 (Ref. 8, Table I, and our unpublished observations). As previously noted (8), this indicates that the C67 residue is not essential to the expression of the disease phenotype. Because the transgene copy number in this line is lower than any other disease-prone line, it is unclear whether the milder phenotype is related to the lower gene copy number, to functional differences between mutant and wild-type B27 (e.g., altered peptide recognition (4)), or to both. Others have speculated that this line may be disease-prone despite the relatively low gene copy number because of the low hβ2m gene copy number and resultant high ratio of B27 to hβ2m, with a consequent increased propensity of the B27 product to misfold (14). However, although this line was confirmed to show a slow rate of H chain folding (Fig. 9B), low hβ2m levels seem unlikely to explain the milder phenotype, because we have observed that disease severity is actually significantly increased in F1 offspring of a cross between the L.33-3 line and a line expressing only hβ2m (our unpublished observations).

The HLA-B7 transgenic line, which is comparable in gene copy number and level of expression to disease-prone B27 transgenic lines (22), shows no evidence of disease compared with nontransgenic LEW rats. This line also showed negligible dimer formation or binding to BiP. Thus, when all of the lines that we studied are considered, a correlation emerges between disease susceptibility and severity on the one hand and B27 H chain misfolding, ER accumulation, dimer and multimer formation, and binding to BiP on the other, assuming that there is also a threshold effect that spares the 21-4L line. The extent to which any of these processes play a role in disease remains to be determined.

In conclusion, our data suggest that the HLA-B27 H chain has an intrinsic propensity to assemble inefficiently, form multimers, and bind to BiP that is relatively independent of the Cys67 residue. The data also suggest that this propensity is closely correlated with the pathogenesis of B27-associated inflammatory disease in vivo. These data do not exclude the possibility that the peptide-binding specificity of B27 may play a major role in disease pathogenesis, and continued investigation along both lines seems fully warranted.

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References


