Formation of HLA-B27 Homodimers and Their Relationship to Assembly Kinetics

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The human HLA-B27 class I molecule exhibits a strong association with the inflammatory arthritic disorder ankylosing spondylitis and other related arthropathies. Major histocompatibility complex class I heavy chains normally associate with β2-microglobulin and peptide in the endoplasmic reticulum before transit to the cell surface. However, an unusual characteristic of HLA-B27 is its ability to form heavy chain homodimers through an unpaired cysteine at position 67 in the peptide groove. Homodimers have previously been detected within the ER and at the cell surface, but their mechanism of formation and role in disease remain undefined. Here we demonstrate, in the rat C58 thymoma cell line and in human HeLa cells transfected with HLA-B27, that homodimer formation involves not only cysteine at position 67 but also the conserved structural cysteine at position 164. We also show that homodimer formation can be induced in the non-disease-associated HLA class I allele HLA-A2 by slowing its assembly rate by incubation of cells at 26 °C, suggesting that homodimer formation in the endoplasmic reticulum may occur as a result of the slower folding kinetics of HLA-B27. Finally, we report an association between unfolded HLA-B27 molecules and immunoglobulin-binding protein at the cell surface.

The major histocompatibility complex (MHC)1 class I molecule HLA-B27 is strongly associated with development of the inflammatory arthritic disease ankylosing spondylitis (AS) and other human spondyloarthropathies (SpAs) (1, 2). Direct evidence supporting a role for HLA-B27 in the disease process has come from transgenic rats, which, under appropriate conditions, develop inflammatory arthritic disease with similar characteristics to human SpAs (3). HLA-B27 when expressed as a transgene in mice can also increase the incidence of a naturally occurring form of inflammatory arthritic disease, referred to as ankylosing spondylitis (4).

The precise role that HLA-B27 plays in disease onset and progression remains undetermined. Several hypotheses have been formed to account for the strong association between HLA-B27 and SpAs (5–8). The arthritogenic peptide hypothesis, for example, proposes that AS is a consequence of the antigen presentation function of HLA-B27. The presentation of pathogen-derived antigens that mimic self peptides presented by HLA-B27 would activate autoreactive T cells (6). Alternatively, it has been suggested that SpAs may not be the result of antigen presentation by HLA-B27 but may arise because of aberrant folding of HLA-B27 molecules within the environment of the endoplasmic reticulum (ER) (9). Support for the idea that HLA-B27 misfolding can contribute to inflammatory arthritis has come from transgenic mice expressing either HLA-B27 (10) or endogenous MHC class I molecules that are unable to assemble for lack of β2-microglobulin (11), which develop a form of spontaneous inflammatory arthritis. In the transgenic rat model, disease occurs only in rats with a transgene copy number above a certain threshold (12), a situation that may potentially promote polypeptide misfolding. It is therefore possible that SpAs could be categorized alongside the increasing number of diseases now recognized to be caused by protein misfolding that have been referred to as ER storage or conformational diseases (13, 14).

SpAs could therefore be related to the intracellular assembly of HLA-B27, a process that normally involves a series of interactions with ER-resident chaperones that ensure appropriate folding (15). Newly synthesized MHC class I heavy chains associated with calnexin, which, upon β2-microglobulin association, is displaced by calreticulin (16–18). Both calnexin and calreticulin can recruit the oxido-reductase ERp57 into these early folding intermediates (19–22). Upon calreticulin association, the partially folded MHC class I molecule associates with the transporter associated with antigen processing (TAP) via the MHC class I-specific accessory molecule tapasin, thus forming the peptide loading complex (18). As part of the peptide loading complex, tapasin is disulfide-bonded to ERp57 (23, 24), and its function promotes the binding of optimal peptides by MHC class I molecules (25, 26).

HLA-B27 exhibits several unusual characteristics. It displays a partially tapasin-independent route of peptide acquisition, which can lead to the binding and presentation of low affinity peptides and the formation of unstable cell surface molecules (27). Within the ER, HLA-B27 exhibits an enhanced susceptibility to undergo ER-associated degradation (28), a pathway utilized by misfolded MHC class I heavy chains (29). Enhanced ER-associated degradation of HLA-B27 appears to
be associated with slow folding kinetics, which is, at least in part, influenced by the composition of the B pocket of the peptide binding groove (28).

A striking feature of HLA-B27 that has recently received much attention is its ability to form heavy chain homodimers (HC-dimers). In vitro folding studies revealed the capacity of HLA-B27 heavy chains to form HC-dimeric structures via a disulfide bridge at the relatively rare unpaired cysteine at position 67 (Cys67) in the α1 helical region of the peptide binding groove (30). Furthermore, two species of HC-dimers have been detected, one within the ER and another at the cell surface (31, 32). These populations of HC-dimers appear to be distinct from each other, since cell surface HC-dimers arise from recycling of HLA-B27-β2m-microglobulin complexes through an endocytic compartment (31), whereas ER-resident HC-dimers do not appear to transit the secretory pathway (32). Another evident feature of these studies was the detection of several HC-dimer structures by SDS-PAGE both in the presence (32) and absence of Cys67 (31). Furthermore, other HLA alleles that do not possess an unpaired Cys67 (e.g., HLA-B7) can form HC-dimers (32, 33), raising the possibility that cysteine residues other than Cys67 could be involved in HC-dimerization.

HC-dimers therefore have two potential ways in which they may be involved in SpAs: mispresentation of peptides and subsequent immune recognition of the HC-dimer structures at the cell surface (34–37), or, alternatively, HC-dimers within the ER may induce ER stress responses (9). However, it remains undetermined how the assembly characteristics of HLA-B27 are related to the formation of HC-dimers and whether these features play a direct role in SpA pathogenesis.

The rat model for AS is the best characterized with respect to disease pathology (38). To complement studies of HLA-B27 in the transgenic rat model of AS, we have undertaken a study of the assembly of the disease associated HLA-B*2705 allele expressed in the rat C58 thyoma cell line. By mutating the conserved structural cysteines of HLA-B27, we demonstrate that HC-dimer formation not only involves Cys67 but can also involve the structural cysteine at position 164 (Cys164). The formation of HC-dimers is not due simply to the presence of Cys67, since the introduction of Cys67 into the non-disease-associated HLA-A2 molecule did not lead to HC-dimer formation. However, HC-dimer formation appears to be related to the assembly rate of HLA-B27. Following incubation at 26 °C, HLA-A2 can adopt similar folding kinetics to HLA-B27 and can form HC-dimeric structures. In addition, we demonstrate that residues flanking position 67 can influence the rate of assembly and contribute to HC-dimer formation. Finally, we provide evidence that unfolded HLA-B27 associates with immunoglobulin-binding protein (BiP) at the cell surface.

**EXPERIMENTAL PROCEDURES**

**DNA and Cell Lines**—The C58 thyoma cell line was maintained in RPMI 1640 supplemented with 5% fetal calf serum. CS.B27, B27.C67S, B27.C101S, B27.C164S, C58.A2, A2.C101S, A2.C164S, A2.C203S, and A2.C259S cells were generated by electroporation of cDNA in vector pCR3 (Invitrogen) with selection in 1 mg/ml G418. B+2705 and A+2010 cDNAs were subjected to directed mutagenesis using the QuiKChange (Stratagene) methodology and sequenced to confirm mutagenesis. T2 and Daudi cell lines were maintained in RPMI 1640 supplemented with 5% fetal calf serum. Transfectant T2.B67 (expressing B+2705) was a gift from Louise Boyle and Hill Gaston (Cambridge, UK). HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and were transiently transfected using 4 μl of Fugene 6 (Roche Applied Science) per well of a 24-well plate, following the manufacturer’s instructions.

**Antibodies**—Monoclonal antibodies and antisera used were as follows: pK (anti-Sv5 tag), ME1 (recognizing folded HLA-B molecules (39)), BB7.2 (anti-HLA-A2 (40)), HC10 (recognizing unassembled HLA-B and -C (41)), anti-IERP57 antisera (courtesy of N. Bulleid, Manchester, UK), anti-calnexin antisera (courtesy of D. Williams, Toronto, Canada), and SPA 926 anti-BiP antisera (Stressgen).

**Immunoblotting of Cell Lysates**—Cell lysates were prepared in 1% Nonidet P-40 lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1× complete protease inhibitors (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 10 mM N-methylmaleimide (NEM) (Sigma)). Lysates were analyzed by 8% SDS-PAGE, blotted onto nitrocellulose membranes (DuPont), blocked in 5% powdered milk in 1× phosphate-buffered saline plus 0.1% Tween, and probed overnight with relevant antibodies. Detection was performed with horseradish peroxidase-conjugated anti-i light chain IgG (Caltag) or horseradish peroxidase-conjugated monoclonal anti-rabbit IgG (Sigma) and visualized with Super signal West Pico and Femto chemiluminescence reagents (Pierce).

**Immunoprecipitations**—Pulse-chase, lysis, immunoprecipitations, and electrophoresis were performed as previously described (42, 43). Briefly, 5 × 106 cells were lysed on ice for 15 min, washed twice with 1× Nonidet P-40 lysis buffer, and probed as described below by immunoprecipitation with pK anti-Sv5 antibody and analyzed nonreduced on 8% SDS-PAGE gels, followed by immunoblotting.

**Cell Surface Biotinylation**—1 × 106 cells were suspended in 1 ml of phosphate-buffered saline containing 1 mg/ml sulfoethylisocyanide biotin (Sigma) and incubated at 4 °C for 20 min. Cells were then washed in phosphate-buffered saline and lysed in detergent buffer supplemented with 10 mM iodoacetamide (IAA) or NEM. Immunoprecipitates were transferred to nitrocellulose and probed with Extravidin (Sigma), followed by development with chemiluminescence reagents as above.

**RESULTS**

**Cysteines 67 and 164 Influence Homodimer Formation**—Previously, Cys67 has been implicated in HC-dimer formation in both in vitro folding experiments and during in vivo expression (30–32). In vivo studies intriguingly detected several HC-dimer species (31, 32). We therefore investigated whether the conserved structural cysteine residues at positions 101, 164, 203, and 259, which form intrachain disulfides, could also influence HC-dimer formation. Each of these cysteine residues was mutated to serine in an HLA-B+2705 (B27) cDNA that was additionally C-terminally tagged with the Sv5 epitope and expressed in the rat C58 thyoma cell line. NEM-treated whole cell lysates were run on SDS-PAGE and immunoblotted with the anti-Sv5 epitope tag antibody pK. HC-dimer structures were detected in wild type B27, C101S, and C259S cells (Fig. 1A, non-reduced panel). HC-dimers were essentially undetectable in C67S cells, although very long exposures revealed a faint band in this region (data not shown), which is in accord with previous reports (31, 32). HC-dimers were also not strongly detected in C164S cells. This observation suggests that HC-dimer formation in the ER is not solely a Cys67,Cys164-mediated event but can involve Cys164.

To further define the role of Cys67 in homodimer formation, we replaced the valine at position 67 (Val67) in HLA-A2 (an HLA allele that is not associated with human SpAs) with cysteine (A2.V67C). We also mutated the conserved cysteine residues in HLA-A2 (A2.C101S, A2.C164S, A2.C203S, and A2.C259S) (Fig. 1B, non-reduced panel). HC-dimers were also not detected in A2.C164S, A2.C203S, and A2.C259S cells. However, a faint HC-dimer structure was detected in the A2.C101S mutant, which may reflect some HC-dimer formation induced through the now unpaired Cys67 residue. These observations suggest that in non-Cys67-containing alleles, the introduction of Cys67 alone is not sufficient for HC-dimer formation and that other factors or residues may influence or be required in this process.

To confirm that the core of our observations were not due to cross-species differences in the assembly of HLA alleles in rat
cells, we transiently expressed cDNAs encoding B27 and mutants C67S and C164S in the human HeLa cell line. Immuno-
blotting cell lysates with HC10 revealed that C67S significantly reduced the number and complexity of HC-dimer for-
mation (Fig. 1C), essentially leaving one band, which was similar to that seen in C58 cells upon long exposure (see above).
Furthermore, expressing increasing amounts of mutant C164S failed to result in the appearance of significant HC-dimers as seen for wild type B27 (Fig. 1D).

Cysteine at Position 67 Slows the Folding of MHC Class I Heavy Chains—The folding rate of HLA-B27 can be influ-
enced by residues making up the B pocket, which include Cys67 (28). We therefore determined the impact of Cys67 on the folding of HLA class I heavy chains in C58 cells. C58.B27, B27.C67S, and C58.A2 cells were metabolically labeled, chased for 0, 1, and 2 h, and then lysed and immunoprecipitated with the confor-
mation-sensitive antibodies ME1 or BB7.2. Following immuno-
precipitation, samples were treated with endo H to assess exit from the ER. HLA-A2 molecules assemble and exit the ER at a faster rate than HLA-B27 molecules (Fig. 2A), replicating the slower folding kinetics seen for HLA-B27 in human cells. It was of interest that in B27.C67S cells a slightly faster rate of ER exit was observed in comparison with wild type B27, with essentially full endo H resistance being attained after 2 h of chase, indicating that Cys 67 affects the assembly of HLA-B27.

To further determine the impact of Cys67 on assembly kinetics, we performed similar experiments comparing B27, B27.C67S, A2, and A2.V67C cells using the conformation-independent pK antibody directed to the C-terminal epitope tag, thus allowing detection of the complete pool of class I molecules and removing any likely influence of the induced mutations upon antibody recognition (44). This approach revealed a large pool of ER-resident (endo H-sensitive) molecules in C58.B27 cells, even after 3 h of chase. By contrast, in B27.C67S cells, this ER-resident pool was not so prevalent. In C58.A2 cells isolation of the full pool of A2 also revealed the ER retention of a pool of A2 molecules, but far less than occurred with B27. Moreover, the introduction of Cys67 into A2 molecules resulted in a partial slowing of its exit from the ER (compare the amount of endo H resistant material at 1 h of chase). Taken together,
these results indicate that the presence of Cys67 significantly slows the assembly and/or exit of a class I molecules from the ER.

Reduced Temperature Enhances and Induces Homodimer Formation in HLA-B27 and HLA-A2, Respectively—The introduction of cysteine at position 67 and the resulting small reduction in folding kinetics in HLA-A2 is not sufficient for HC-dimer formation (Figs. 1 and 2). The pulse-chase experiment in Fig. 2B suggests that A2.V67C can, moderately, fold more efficiently than wild type HLA-B27. We therefore attempted to further reduce the folding kinetics of A2 by incubating cells at 26°C. Cells incubated overnight at 26°C were metabolically labeled, chased, and immunoprecipitated with pK antibody. Under these conditions, the ER exit of A2 resembled closely that of wild type B27 (Fig. 2, compare B and C).

We next determined the effect of 26°C treatment on HC-dimer formation in B27, A2, and their respective mutants (Fig. 3). Immunoblotting of whole cell lysates revealed that 26°C treatment enhances the presence of B27 HC-dimers in wild type and mutant B27 molecules (Fig. 3A). Surprisingly, 26°C treatment also induced C67S to form more obvious HC-dimer structures.

Similar treatment of A2-expressing cells unexpectedly revealed the formation of HC-dimer structures in varying amounts (Fig. 3B). Most notably, the presence of V67C now permitted enhanced formation of HC-dimers compared with wild type A2. In order to eliminate the possibility that the observed high molecular weight structures were not the result of enhanced interactions between ERp57 and class I (45), the blot was stripped and reprobed with anti-ERp57 antibodies. None of the bands observed in Fig. 3B could be ascribed to ERp57-class I complexes (data not shown). Taken together, these results suggest that the folding efficiency of a class I molecule in the ER can influence its ability to form into HC-dimer structures.

Thus, B27, due to its slower folding, may be predisposed to the formation of HC-dimers in the ER.

We also characterized the rate of HLA-A2 HC-dimer formation at 26°C. A2, A2.V67C, A2.C101S, A2.C164S, and A2.C203S cells were incubated at 26°C for the times stated. After each time point, cells were treated with NEM, lysed, and analyzed by immunoblotting with pK antibody. 0 time point reflects HC-dimerization at 37°C. B, cell lysates of T2, T2.B27, and Daudi cells incubated at 37 or 26°C overnight were immunoblotted with antibody HC10.

Effect of Reduced Temperature and Cys67 upon Interactions with ER Chaperones—Whereas we failed to detect class I-ERp57 complexes in the immunoblotting of whole cell lysates shown in Fig. 3, we were interested to know whether 26°C treatment could indeed enhance class I-ERp57 and other chaperone interactions. In previous work, we have only been able to...
Interestingly, 26°C treatment appeared to slightly enhance the reactivity of Cys67 into HLA-A2 failed to induce significant HC-dimer formation at physiological temperatures, suggesting that additional residues might be required. It has been reported that the reactivity of Cys67 is also influenced by Lys70 (46). Therefore, in addition to Cys67, the following mutations were introduced into A2.V67C to mutate flanking residues to those of HLA-B27: (a) R65Q plus K66I (referred to as A2.QIC) and (b) H70K plus S71A (referred to as A2.CKA). Whole cell lysates of C58 cells expressing these mutants were immunoblotted for HC-dimers (Fig. 6A). In both cases, HC-dimers were detected at levels above those of A2.V67C cells, suggesting that residues flanking Cys67 play a role in HC-dimer formation. Pulse-chase analysis of these mutants further revealed a reduction in the amount of endo H-resistant molecules detected after 1 and 3 h of chase (Fig. 6B). Thus, again, the data support a correlation between slow folding kinetics and HC-dimer formation.

Cell Surface Expression of Homodimers—Cell surface HLA-B27 HC-dimers have been reported in various cells (31, 32). To determine their presence in rat cells, we performed surface biotinylation of C58.B27. Lysates were prepared in the presence of either IAA or NEM, followed by immunoprecipitation with the monoclonal antibody HC10. Lysis in NEM significantly lowered the amount of HC-dimers detected on C58.B27 cells compared with IAA, suggesting that the formation of some cell surface HC-dimers may result from postlysate effects due to incomplete alkylation (Fig. 7A). Upon reduction of the samples, a single species of approximate Mr 70,000 remained in both IAA- and NEM-treated samples. We considered that the extra species may be cell surface BiP, a normally ER-resident chaperone that frequently can be detected at low levels at the cell surface (47). To test this hypothesis, cell surface biotinylated C58 and C58.B27 NEM lysates were prepared and immunoprecipitated with an anti-BiP antibody (Fig. 7C). In these reduced samples, bands with identical SDS-PAGE migration were isolated by the anti-BiP antibody and in association with HC10-reactive B27 molecules. Fluorescence-activated cell sorting analysis also revealed low levels of cell surface BiP (not shown). To formally identify the Mr 70,000 species, we performed large scale immunoprecipitations of C58.B27 cells with HC10 and subjected the relevant gel band to mass spectrometric analysis, which positively confirmed its identity as BiP (data not shown).

Recently, a new theory was postulated to be involved in human SpA disease (7, 9, 36). Recent studies, however, have demonstrated multiple HC-dimeric structures, which may form independently in the ER during the early stages of biosynthesis or at the cell surface following unfolding of normal HLA-B27 molecules, possibly involving trafficking through an endocytic compartment. The existence of HC-dimers has been called into question (48). In our own experience, we find that some HC-dimers may be formed as a result of inefficient sample alkyla-
tion, especially those deriving from the cell surface. Nevertheless, even after prolonged incubation with the cell-permeable alkylating agent NEM, we are still able to detect the ER form of HC-dimers. In this study, we have focused on the ER form by mutating structural cysteine residues in HLA-B27 and -A2 molecules. The unusual cysteine at position 67 of HLA-B27 has been a major focus for investigation. Importantly, Cys 67 is a feature that is shared by a relatively small cohort of class I alleles, including subtypes of HLA-B14, -B15, -B38, -B39, and -B73 (49). It is unknown whether these subtypes also form HC-dimeric structures, although it is interesting to note that HLA-B39 is also reportedly linked to SpA (50, 51). In this report, we have demonstrated that HC-dimer formation can be prevented by replacement of the structural Cys164 residue. This raises the possibility that HC-dimers may be able to form through the interaction of Cys67-Cys164 disulfides on adjacent molecules and would lead to a significant alteration of the proposed structure for back-to-back HC-dimers with unfolded α helices (52). With respect to this observation, it is important to note that, as might be expected, all of the structural cysteine mutants created in both B27 and A2 in this study (i.e. C101S, C164S, C203S, and C259S) fail to assemble fully into structures recognized by conformational antibodies and also fail to exit the ER. They thus represent exclusively the ER form of HC-dimers, of which the relationship to cell surface HC-dimers remains to be fully established. As previously reported (32), we also find that HC-dimer structures in C58.B27 cells have a pulse-chase phenotype indicative of retention in the ER.

Our observation that incubation of cells at reduced temperature enhances (in HLA-B27) and induces (in HLA-A2) HC-dimer formation suggests that dimerization of MHC class I heavy chains is an inherent risk for class I molecules that fold with slow kinetics. It is interesting to note that Nößner and Parham (33) also detected MHC class I HC-dimeric structures when HLA-B*0702 was expressed in C1R cells, in which the assembly rate was considerably slower than in normal B cells. Such structures would not normally be observed if MHC class I heavy chains undergoing folding did not accumulate and if they assembled rapidly and stably, which would normally be the case for HLA-A2 but not for HLA-B27. Incubation of C58.B27 cells with proteasome inhibitors and with reagents that block ER-associated degradation leads to an accumulation of HC-dimers, suggesting that the quality control processes in

FIG. 6. Residues flanking Cys67 influence homodimer formation and rate of folding. A, lysates from NEM-pretreated A2, A2.V67C, A2.QIC, and A2.CKA cells were analyzed by immunoblotting with pK antibody. B, cells were metabolically labeled; chased for 0, 1, and 3 h; immunoprecipitated with pK antibody; treated (+) or untreated (−) with endo H; and analyzed by SDS-PAGE.

FIG. 7. Homodimer expression at the cell surface. C58 and C58.B27 cells were surface-biotinylated, lysed in the presence of IAA (A) or NEM (B), immunoprecipitated with HC10 antibody, and analyzed (reduced and nonreduced) by immunoblotting with extravidin-horseradish peroxidase. C, surface-biotinylated cells were lysed in NEM and immunoprecipitated with ME1, HC10, and anti-BiP antibody and analyzed as described.

*A. N. Antoniou and S. J. Powis, unpublished observations.*
the ER targets these structures for degradation.

Whereas the majority of our studies presented here have focused on the assembly of HLA-B27 in a rat cell line, we have also been able to demonstrate several key points in a human cell line. Crucially, neither C67S or C164S mutants form the HC-dimer pool as seen for wild type B27. Furthermore, we also show that HC-dimers are formed in human cells after incubation at reduced temperature. Our observations are therefore not restricted by use of the rat C58 cell line, although we have no doubt that some differences in the assembly pathways do indeed occur between different species.

We have demonstrated that Cys67 can also influence the rate of folding of a class I molecule within the ER. This may, in part, be due to peptide binding during the folding process as Cys67 can influence the peptide repertoire (53). Our data suggest that by removing Cys67, HLA-B27 folding becomes more efficient; however, de Castro and co-workers (53) suggest that C67S molecules are relatively unstable at the cell surface, which may potentially imply a lack of full quality control in the ER for these molecules.

The residues surrounding Cys67 can also influence the formation of HC-dimers. Interestingly, only HLA-B27 and -B73 have a Lys70 residue that confers enhanced chemical reactivity to Cys67 (46). However, it has not been documented how Lys70 affects HC-dimer formation. We have shown that Lys70 (and possibly Ala70) can influence HC-dimer formation when substituted into HLA-A2. Residues Gln65 and Ile66 can also enhance HC-dimer formation. This latter observation cannot immediately be reconciled to previous studies on the Cys67 region, but the demonstration that A2.QIC has a slow assembly rate further implicates assembly kinetics as a factor in HC-dimer formation.

How can slow folding predispose to HC-dimer formation? A slow folding rate would presumably increase the local concentration of partially folded/unfolded HLA-B27 molecules, leaving exposed unpaired cysteine residues, which could lead to aberrant disulfide bond formation. Increased local concentrations of MHC class I molecules with unusual unpaired cysteine residues (e.g. HLA-G) has been suggested as the mechanism promoting HC-dimerization (54). Intriguingly, a recent study revealed that AS patients can express elevated levels of HLA-B27 (55), which, if reflected in the ER, could promote aberrant HLA-B27 folding.

A further possibility is that the slow folding of HLA-B27, in combination with Cys67, enhances the interaction with ER chaperones. HC-dimerization in the ER could therefore be the result of ERp57-mediated oxidoreductase activity. In support of this idea, we have demonstrated the presence of ERp57-B27 complexes, whereas we failed to detect significant ERp57-A2 complexes. This may suggest that prolonged interaction with ERp57 during early assembly events is involved in HC-dimer formation. However, we also detected ERp57-B27.C67S complexes, which would suggest that this is not the critical event in HC-dimer formation. By introducing Cys67 into HLA-A2, we were able to detect ERp57-A2 complexes, but only at reduced temperature. Therefore, we suggest that Cys67 in combination with a slow folding rate can enhance the MHC class I heavy chain-ERp57 interaction. A more stringent test of the requirement for ERp57 in HC-dimer formation will require an ERp57-deficient cell line or possibly small interfering RNA inhibition studies.

BiP is one of the most abundant chaperones in the ER and is known to associate transiently with many newly synthesized (56, 57), unfolded and mutant proteins (58, 59). Indeed, it has been demonstrated that proteins that fold more slowly or unstably are preferentially recognized by BiP (60). Our studies support this observation in the case of BiP-HLA class I molecule associations. BiP is also a key component of the unfolded protein response (61, 62), acting as the main sensor of ER stress and keeping the main effector molecules of the ER stress pathway (ATF6, IRE1, and PERK) in check under nonstressed conditions (62, 63). Upon sensing ER stress, BiP dissociates from these effector molecules, presumably to perform its role as a chaperone. Dissociation of these effector molecules, allowing unfolded protein response to take place. The strong BiP-B27 interaction could support the hypothesis that SpA is caused by HLA-B27 misfolding (9). Interestingly, BiP has been shown to be up-regulated in synovial fluid mononuclear cells derived from SpA patients (64). Furthermore, we report here the association of BiP on the cell surface with unfolded or partially folded B27 molecules. Cell surface expression of BiP has been reported previously and could reflect escape from the quality control process, resulting in sorting to the wrong compartment (47, 65–67). Several recent studies have reported an unusual population of CD4+ CD28– NK-like T cells in SpA (68). The structure that these cells recognize has not been defined, but one possibility is that they recognize HC-dimer structures on the cell surface. Although we cannot yet distinguish whether BiP is associating with unfolded B27 monomers or HC-dimers or both, it is potentially worth considering whether a BiP-B27 structure may be being recognized by the immune system.

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