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ORIGINAL RESEARCH COMMUNICATION

The MHC Class I Heavy Chain Structurally Conserved Cysteines 101 and 164 Participate in HLA-B27 Dimer Formation

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Abstract

Aims: The human leukocyte antigen (HLA)-B27 is strongly associated with a group of inflammatory arthritic disorders known as the spondyloarthropathies (SpAs). The unusual biochemistry of HLA-B27 has been proposed to participate in disease development, especially the enhanced ability of HLA-B27 to form several heavy chain-dimer populations. HLA-B27 possesses three unpaired cysteine (C) residues at position 67, 308, and 325, in addition to the four conserved cysteine residues at p101, 164, 203, and 259. C67 was proposed to participate in dimer formation of recombinant HLA-B27 protein and *in vivo* heavy chain-dimers. However, the structurally conserved C164 was demonstrated to participate in endoplasmic reticulum (ER) resident heavy chain-dimer formation. We therefore wanted to determine whether these aggregates involve cysteines other than C164 and the basis for the difference between the observed heavy chain-dimer species. ***Results:*** We determined that C164 and C101 can form distinct dimer structures and that the heterogenous nature of heavy chain-dimer species is due to differences in both redox status and conformation. Different HLA-B27 dimer populations can be found in physiologically relevant cell types derived from HLA-B27-positive patients with inflammatory arthritis. In addition, HLA-B27 dimer formation can be correlated with cellular stress induction. ***Innovation:*** The use of both mutagenesis and manipulating cellular redox environments demonstrates that HLA-B27 dimerization requires both specific cysteine–cysteine interactions and conformations with differing redox states. ***Conclusion:*** HLA-B27 heavy chain-dimerization is a complex process and these findings provide an insight into HLA-B27 misfolding and a potential contribution to inflammatory disease development. *Antioxid. Redox Signal*. 16, 33–43.

Introduction

HLA-B27 is a Major Histocompatibility Complex (MHC) class I molecule that presents peptides to cytotoxic $CD8^+$ T cells and is recognized by natural killer (NK) cell receptors. HLA-B27 is a member of the human leukocyte antigen HLA-B family of MHC class I alleles and is strongly associated with a group of inflammatory arthritic disorders [*i.e.,* the spondyloarthropathies (SpAs) (6, 22)].

MHC class I molecules are composed of a heavy chain noncovalently associated with a beta-2-microglobulin (β2m) light chain and an 8–13 amino acid long peptide. MHC class I

Innovation

Using a combination of mutagenesis and redox chemistry, the authors demonstrate that HLA-B27 dimerization is a complex process, involving the structurally conserved residues C101 and C164. HLA-B27 dimers exist as multiple species in ankylosing spondylitis patients, with differing redox states and/or conformations. HLA-B27 dimer expression can be correlated with UPR induction, implicating cellular stress pathways in the pathology associated with HLA-B27 mediated inflammatory arthritis.

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molecules fold within the oxidizing environment of the endoplasmic reticulum (ER) lumen via a series of transient interactions with ER resident chaperones (4), leading to the formation of the peptide loading complex (PLC) whose main function is the optimization of the peptide cargo for presentation to cytotoxic T lymphocytes (9, 10, 27). HLA-B27 has an enhanced tendency to misfold which is influenced by residues in the antigen binding groove (17) and can exist as different conformers within the ER (13). HLA-B27 also forms heavy chain-dimers (1).

MHC class I heavy chains possess four conserved cysteine (C) residues, which form two structurally important disulfide bonds within the α2-domain between C101–C164 and the α3-domain between C203–C259. In addition, HLA-B27 expresses three additional unpaired cysteines at positions 67, 308, and 325. It was originally proposed that C67, located within the antigen binding groove, participated in the dimerization of HLA-B27 heavy chains (1), of which there are two populations, one cell surface and one ER-resident, which form independently. (5). We have demonstrated that dimerization within the ER requires the conserved C164 (3) and the process is related to heavy chain misfolding and maturation kinetics (3, 11, 17).

Analysis of HLA-B27 dimers indicates a nonhomogenous population, therefore we wanted to; (a) determine whether these aggregates involve cysteines other than C164, and (b) determine the basis for the difference between the observed heavy chain-dimer species. In this study, we show that C164 is important in ER HLA-B27 dimer formation. The absence of both C164 and C67 allows dimerization via C101, whilst the absence of C101 and C164 does not lead to C67–C67 interactions. These observations suggest a hierarchy and/or preference of disulfide bonding between the α2-domain cysteines and the unpaired C67. Using reducing and oxidation reactions, we also demonstrate that HLA-B27 forms conformationally distinct dimeric species. The differential susceptibility of HLA-B27 dimers to reducing and oxidizing agents demonstrates for the first time that *in vivo* HLA-B27-dimers have altered redox states.

Results

The α2-domain cysteines C101 and C164 participate in heavy chain dimerization

Predicted models of HLA-B27 dimers have postulated a degree of unfolding of the antigen-binding groove to allow C67–C67 interactions to occur (16). We hypothesized that other cysteine residues could participate in dimer formation if such unfolding events did take place. Mutagenesis studies revealed a role for C164 (3), however, the HLA-B27.C164S mutant may remain in a partially folded state, preventing C67–C67 interactions. To address this possibility, both the conserved α2(C101–C164) and α3(C203–C259) domain cysteines of HLA-B*2705 were mutated to serines(S).

Both HLA-B27.C101S–C164S and HLA-B27.C203S–C259S (V5 C-terminally tagged) mutants were expressed in the C58 rat thymoma cell line but were not detected at the cell surface (data not shown), suggesting these two molecules do not attain a fully folded state. To determine the role of the conserved cysteines in dimer formation, HLA-B27.C101S–C164S and HLA-B27.C203S–C259S expressing cell lines were pretreated with the alkylating agent N-methylmalemide (NEM) to trap and maintain any transient disulfide bonded species. Immunoblotting revealed that HLA-B27-dimers were ablated in the C101S–C164S mutant (Fig. 1A). The C203S–C259S mutant retained the ability to dimerize, indicating that the α2-domain cysteines are required for dimerization.

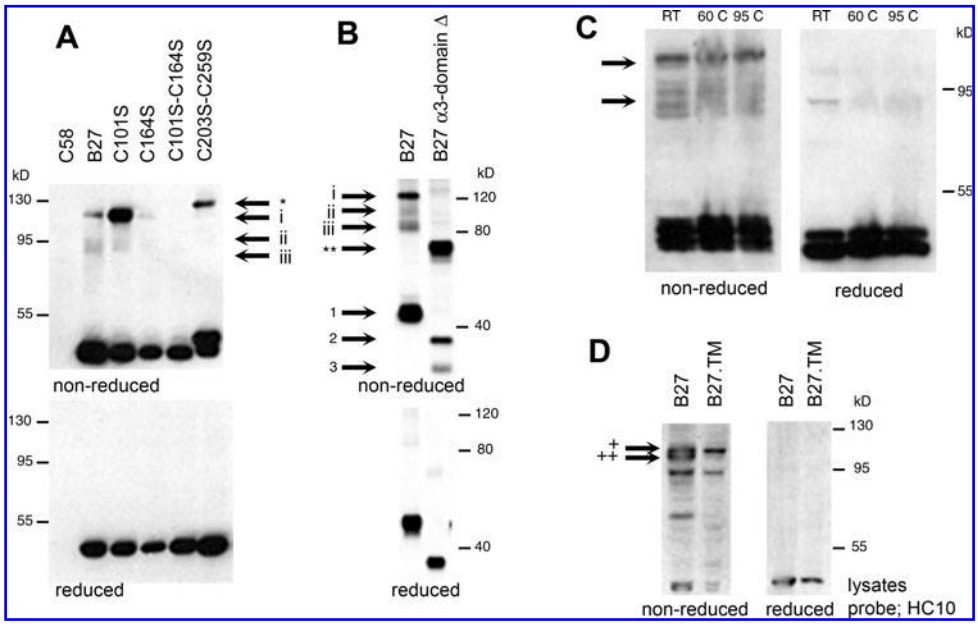

FIG. 1. The structurally conserved cysteines C101 and C164 participate in HLA-B27 heavy chain dimer formation. (A) Lysates from NEM-treated untransfected C58 cells or cells transfected with HLA-B*2705, B27.C101S, B27.C164S, B27.C101S–C164S and B27.C203S–C259S were analyzed by nonreducing (*top panel*) and reducing (*bottom panel*) SDS-PAGE and immunoblotted with the anti-V5 pK antibody. Heavy chain dimers are indicated by *arrows* i, ii, iii, and *. Monomeric heavy chain is seen as a prominent band at approximately 45 kD. **(B)** Lysates from NEM-treated C58 cells transfected with either HLA-B*2705 or B27.Δα3domain, were run nonreduced (*top panel*) and reduced (*bottom panel*) and immunoblotted with anti-V5 pK antibody. Heavy chain dimeric structures are indicated by *arrows* i, ii, iii, and ** under nonreducing conditions. **(C)** CEM cells expressing HLA-B27 were lysed and treated with 8 *M* urea at room temperature, 60°C, and 95°C before analysis by immunoblotting with pK antibody. **(D)** Lysates from NEM-treated C58 cells expressing B*2705 and B27.TM were analyzed by nonreducing and reducing SDS-PAGE and immunoblotted with HC10. Heavy chain dimers of interest are highlighted by *arrows* + and ++.

Immunoblotting of wild-type HLA-B*2705 revealed at least three distinct HLA-B27-dimer populations (Figs. 1A and 1B, arrows i, ii, and iii). A single dimer band was evident in the HLA-B27.C203S–C259S mutant (Fig. 1A, arrow*) suggesting that an intact α3-domain is not required for dimer formation. To confirm this latter observation we constructed an HLA-B27 molecule lacking the α3-domain (Δα3-domain). The HLA-B27.Δα3-domain was not detected at the cell surface (data not shown) and immunoblotting revealed a single distinct high Mw species (Fig. 1B, arrow**) when compared with wild-type HLA-B*2705 (Fig. 1B, arrows i, ii, iii). Under nonreducing conditions, the HLA-B27.Δα3-domain monomer ran as two distinct forms (Fig. 1B, arrows 2, 3) compared to HLA-B*2705 (Fig. 1B, arrow 1), which probably reflects reduced (Fig. 1B, arrow 2) and oxidized (Fig. 1B, arrow 3) conformers at steady state levels.

HLA-B27-dimers could arise from noncovalent interactions; therefore, we pretreated lysates with 8 *M* urea, followed by incubation at increasing temperatures. Immunoblotting revealed that dimers remained intact, strongly suggesting that noncovalent associations play little if any role in HLA-B27 dimerization (Fig. 1C).

To further confirm that the α2 domain cysteines participate in dimerization, we constructed an HLA-B27 molecule lacking all three unpaired cysteines at p67, 308, and 325 (HLA-B27.C67S.C308S.C325S, referred to as HLA-B27.triple mutant (TM)). Immunoblotting with HC10 revealed dimeric structures (Fig. 1D, arrows +, ++), further confirming that C101 and C164 participate in HLA-B27 dimer formation.

Cysteine 164 and 101 can participate in ER resident HLA-B27 heavy chain dimerization

The absence of C164 could allow C67 to form intramolecular disulfide bonds with C101, thus preventing intermolecular C67–C67 and C67–C164 interactions. To address this, we mutated C67 together with C164 and analyzed dimerization. The HLA-B27.C67S–C164S mutant exhibited an enhanced ability to dimerize compared to the HLA-B27.C164S mutant (Figs. 2A and 2B, arrow F1). To determine whether C164 could participate directly in dimerization, an HLA-B27.C67S–C101S mutant was generated. Immunoblotting confirmed C164–C164 mediated dimerization (Fig. 2B, arrows F2, F3, F4).

Very little of the HLA-B27.C101S–C67S and HLA-B27.C164S–C67S could be detected at the cell surface with both ME1 and HC10 antibodies (Fig. 3A, top panel). Intracellular staining with the pK antibody revealed protein levels of each to be within two-fold of each other. Apart from C67S, these mutants do not attain a fully folded state and remain within the ER (Fig. 3A, bottom panel). To determine if these dimers were ER resident, lysates from each cysteine mutant were treated with endoglycosidase H (endo H) which specifically cleaves oligosaccharide additions of ER resident proteins. Each of the high Mw conformers (Fig. 3B, lanes 3, 5, 7, 9, 11, 13) were all sensitive to endo H digestion (Fig. 3B, lanes 4, 6, 8, 10, 12, 14) suggesting they are ER resident and do not attain a mature phenotype (Fig. 3B).

HLA-B27 forms distinct heavy chain dimers with differential susceptibility to reduction

HLA-B27-dimers were composed of at least three different species. To determine if these distinct HLA-B27 dimer

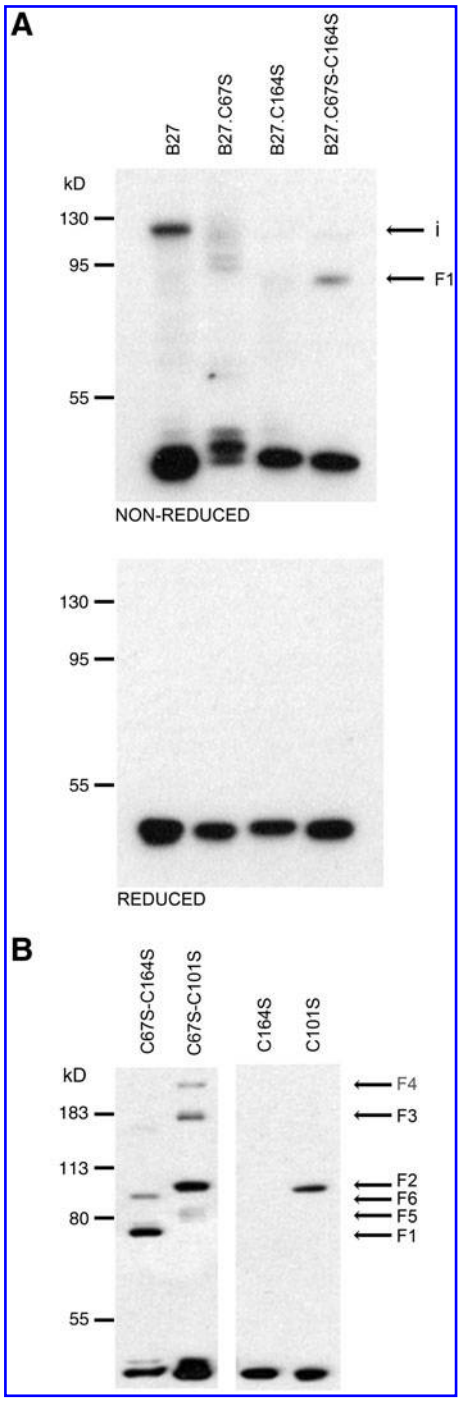

FIG. 2. Cysteine 164 is involved in complex redox reactions with C101 and C67. (A) C58 cells expressing HLA-B27, C67S, C164S, and C67S–C164S were NEM treated prior to lysis. Lysates were analyzed by nonreducing (*top panel*) and reducing SDS-PAGE (*bottom panel*) and immunoblotted with pK antibody. Heavy chain dimers are indicated by *arrow* i and a novel high molecular weight form by F1. **(B)** Lysates from C164S, C67S-C164S were generated as in **(A)**, analyzed by nonreducing SDS-PAGE, but compared to lysates generated from C58 cells expressing C101S and C67S–C101S. The high molecular weight forms of HLA-B27 are indicated by *arrows* F1-F6. The minor high MW populations detected (*arrows* F3–F6), were probed for ERp57, PDI, ERp72, and tapasin, but none of these high MW bands appeared to contain any of these molecules (data not shown).

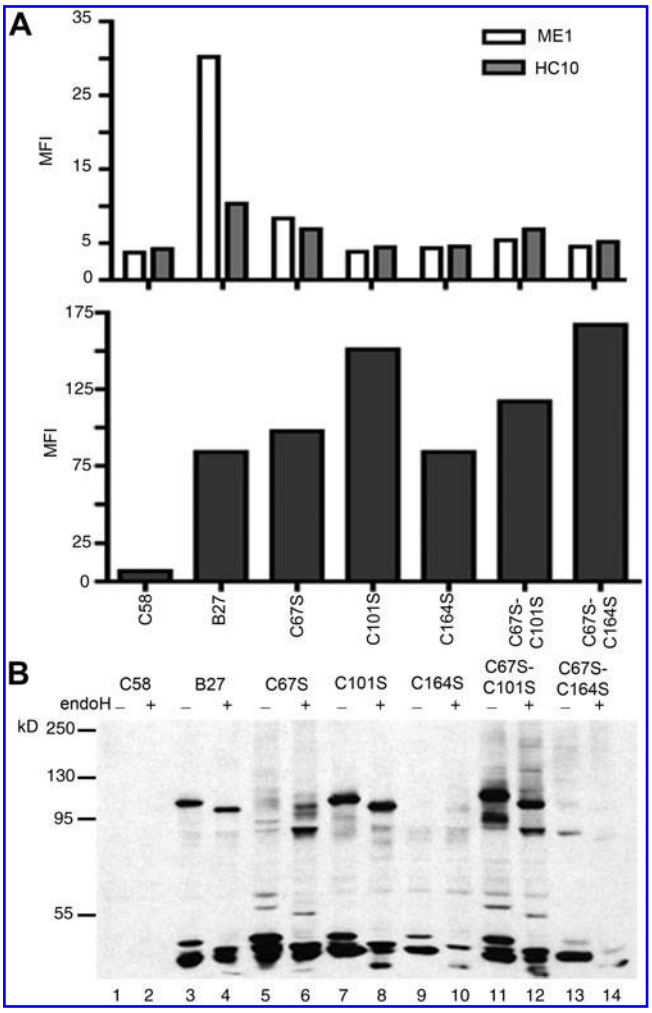

FIG. 3. HLA-B27 heavy chain dimers are predominantly within the endoplasmic reticulum. (A) Untransfected C58 and C58 cells expressing HLA-B27, C67S, C101S, C164S, C67S–C101S, and C67S–C164S were either stained for cell surface HLA-B27 with ME1 or HC10 (*top panel*) or for intracellular expression using pK antibody (*bottom panel*). The mean fluorescence intensities (MFI) were plotted for each cell line. **(B)** Lysates from the cell lines from **(A)** were digested with endoglycosidase H enzyme for 1 h at 37°C, prior to analysis by nonreducing SDS-PAGE and immunoblotting with pK. The decreased MW of the endo H treated heavy chain dimers can be seen in *lanes* 4, 6, 8, 10, 12, and 14 when compared to their respective undigested counterparts in *lanes* 3, 5, 7, 9, 11, and 13, thus indicating that heavy chain dimers do not transit through the secretory pathway.

populations were rat cell line specific (Figs. 1 and 2), we expressed the V5 C-terminally tagged HLA-B*2705 in the human lymphoblastoid CEM cell line. Immunoblot analysis of CEM and C58 cells expressing HLA-B27 revealed that three distinct dimer bands were evident in both cell lines (Fig. 4A, arrows i, ii, and iii). In addition, all three species could be detected in the C58.B27 Sv5 tag expressing cell line with the HC10 antibody (Fig. 4A). To determine whether dimers could be detected at physiological levels of expression, we immunoblotted lysates from two HLA-B27 expressing Epstein Barr virus (EBV) transformed cell lines HOM2 and WEWAKI (19) with HC10, which revealed high MW species (Fig. 4B, arrow +, ++). A similar pattern of dimer complexity was also observed in some, but not all, HC10 immunoblots of lysates of blood monocyte-derived dendritic cells from AS patients, stimulated for up to 3 days in LPS (arrows, Fig. 4C). Thus, the complexity of HC10 reactive bands is also detected in patient-derived samples.

The different dimer populations could be explained by; (a) the heavy chain dimeric populations each exhibiting different redox states, (b) the different redox states being accompanied by conformational changes, or (c) HLA-B27 forming distinct conjugates with other ER resident proteins which participate in MHC class I assembly.

To understand the composition and potential redox differences between these HLA-B27 dimers, we tested their susceptibility to reduction. CEM cells expressing HLA-B*2705 were treated with increasing concentrations of dTT (0–10 m*M*) for 10 min at 37°C before NEM alkylation and lysis. Immunoblotting lysates revealed that dTT treatment did not reduce all dimers (Fig. 4D). Each high MW structure exhibited differential susceptibility to reduction. Band iii was the most susceptible to reduction (Fig. 4D, arrow iii). The disappearance of bands ii and i was accompanied by the appearance of additional bands (Fig. 4D, RED1, 2). The largest migrating HLA-B27dimer (Fig. 4D, arrow i) appears to be the most resistant to reduction. The appearance of both a smear following treatment with 1 m*M* dTT and a larger MW band at 10 m*M* dTT (Fig. 4D, RED2), is indicative of substrates undergoing a series of redox and possibly conformational changes before final reduction.

*High MW conformers are composed of HLA-B*2705 homodimers*

The differential dTT mediated reduction exhibited by the different dimer species could be explained if they were composed of different MHC class I–protein conjugates, each with varying susceptibility to reduction. We took advantage of this observation to probe for known protein substrates that have previously been demonstrated or postulated to interact with MHC class I heavy chains. These included the oxidoreductases ERp57 (2), ERp72 (15), and PDI (18). We also probed for tapasin, which has also been postulated to form direct conjugates with MHC class I heavy chains (8, 20). Any disulfide bonded conjugates with HLA-B27 should exhibit similar high MW complexes with identical susceptibility to reduction. Immunoblotting lysates generated from CEM cells expressing HLA-B*2705 which were incubated with dTT and pretreated with NEM prior to lysis, did not reveal any HLA-B27-ERp57, -ERp72, -PDI, or -tapasin conjugates (Fig. 5A). However, it is possible that due to the very small percentage of the pool of these molecules interacting with MHC class I heavy chains, their detection could be beyond the sensitivity of this experimental system. We thus decided to immunoprecipitate HLA-B27 heavy chains using an assay system capable of detecting HLA-B27-ERp57 conjugates (2, 3). Approximately 5×10^7 CEM cells expressing HLA-B*2705 were each treated with either 0, 1, 5, and 10 m*M* dTT for 10 min at 37°C, followed by NEM alkylation and lysis in 1% NP40 detergent. MHC class I heavy chains were immunoprecipitated with pK antibody, followed by immunoblotting for ERp57. The ERp57–MHC

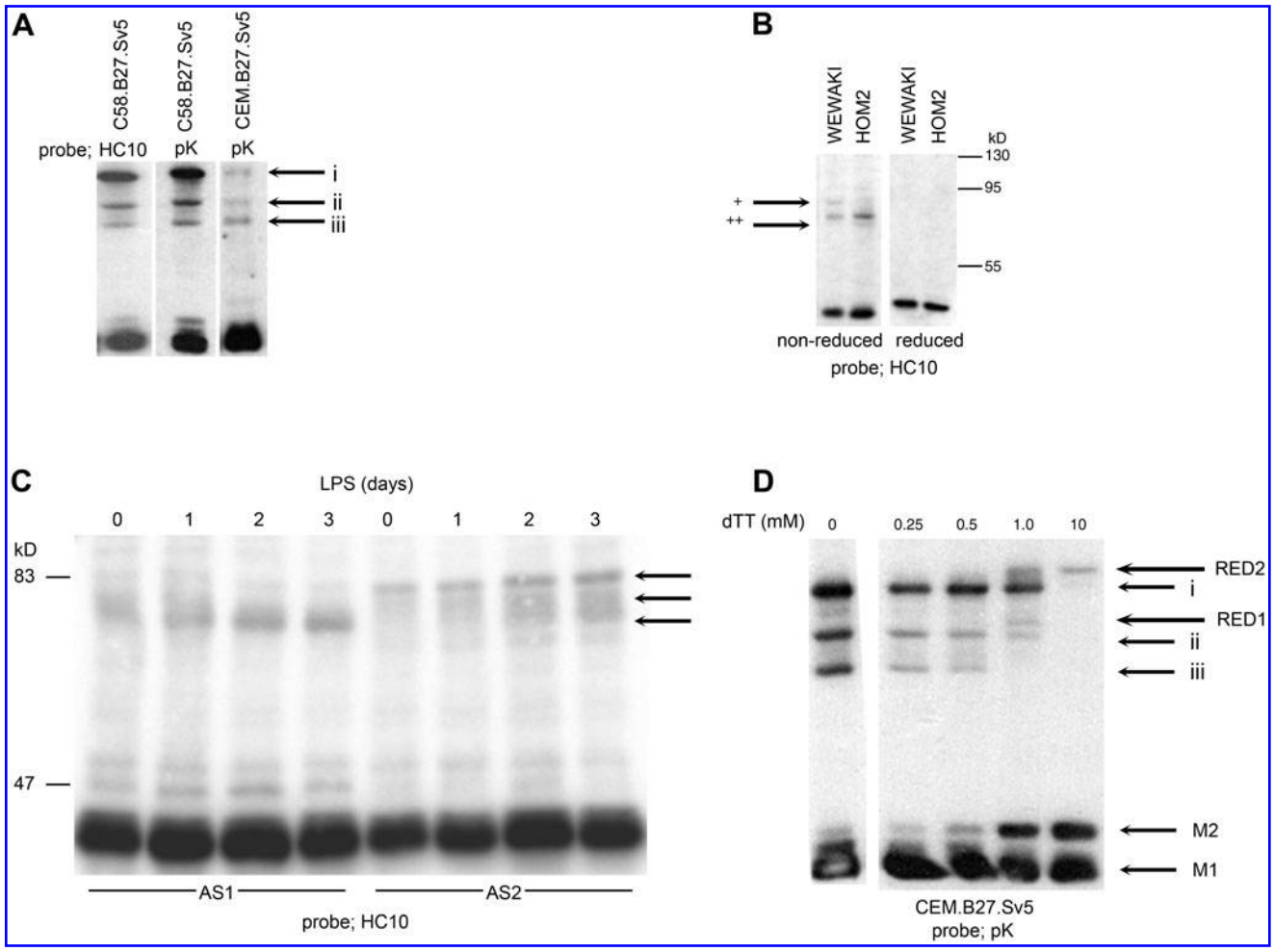

FIG. 4. HLA-B27 heavy chain dimers are common to both rat and human cells and exhibit differential susceptibility to dTT-mediated reduction. (A) Lysates from C58 cells expressing HLA-B*2705 lysates were generated as described in Materials and Methods, analyzed by nonreducing SDS-PAGE and either immunoblotted with HC10 or pK antibodies and compared to lysates immunoblotted with pK from CEM cells transfected with HLA-B*2705. Heavy chain dimers are indicated by *arrows* i, ii, and iii. **(B)** Human EBV cell lines WEWAKI and HOM2, expressing HLA-B27, were NEM treated prior to lysis. Lysates were analyzed by nonreducing and reducing SDS-PAGE and immunoblotted with HC10. High MW structures are indicated by *arrows* + and ++. **(C)** Equal numbers of unstimulated or LPS stimulated monocyte-derived dendritic cells from AS patients 1 and 2 were lysed and immunoblotted with HC10 antibody. Different heavy chain dimeric conformers are indicated by *arrows*. **(D)** Equal numbers of CEM cells expressing HLA-B27 were incubated with increasing concentrations of dTT (0, 0.25, 0.5, 1.0, and 10 m*M*) and incubated at 37°C for 10 min, then NEM treated and lysed. Lysates were analyzed by nonreducing SDS-PAGE and immunoblotted with pK antibody. Heavy chain dimers are indicated by *arrows* i, ii, and iii. Different conformers of heavy chain dimers, which appeared following reduction, are indicated by *arrows* RED 1 and RED 2. Monomeric heavy chain is indicated by *arrows* M1 and heavy chain which is in a reduced state by arrow M2.

class I conjugate was observed to be sensitive to as little as 1 m*M* dTT (Fig. 5B). We were unable to detect any complexes with PDI, tapasin, and ERp72 (data not shown).

HLA-B27 heavy chain dimers each have distinct redox states

The varying gel migration of dimers could be due to differences in redox status or represent molecules that have undergone conformational changes. The faster migration rate of some bands (Fig. 2C, arrows ii, iii) may be indicative of a more oxidized and possibly compact molecular conformation than that represented by band i. To test this, we treated CEM cells expressing HLA-B*2705 with increasing concentrations of the thiol oxidizing agents diamide and 1-chloro-2,4-dinitrobenzene (CNDB), which depletes reduced glutathione (GSH). If the bands migrated according to their different oxidation states, band i (Fig. 2) should be more susceptible to increasing oxidative conditions. Furthermore, by increasing the oxidation conditions, we should also observe the accumulation of the lower MW populations (Fig. 2B, bands ii, iii) representing compact oxidized species. Increased diamide and CNDB concentrations did indeed lead to the disappearance of the upper most high MW band (Figs. 6A and 6B, arrow i), strongly suggesting that this structure is composed of an 'open' conformation whose cysteine residues are more

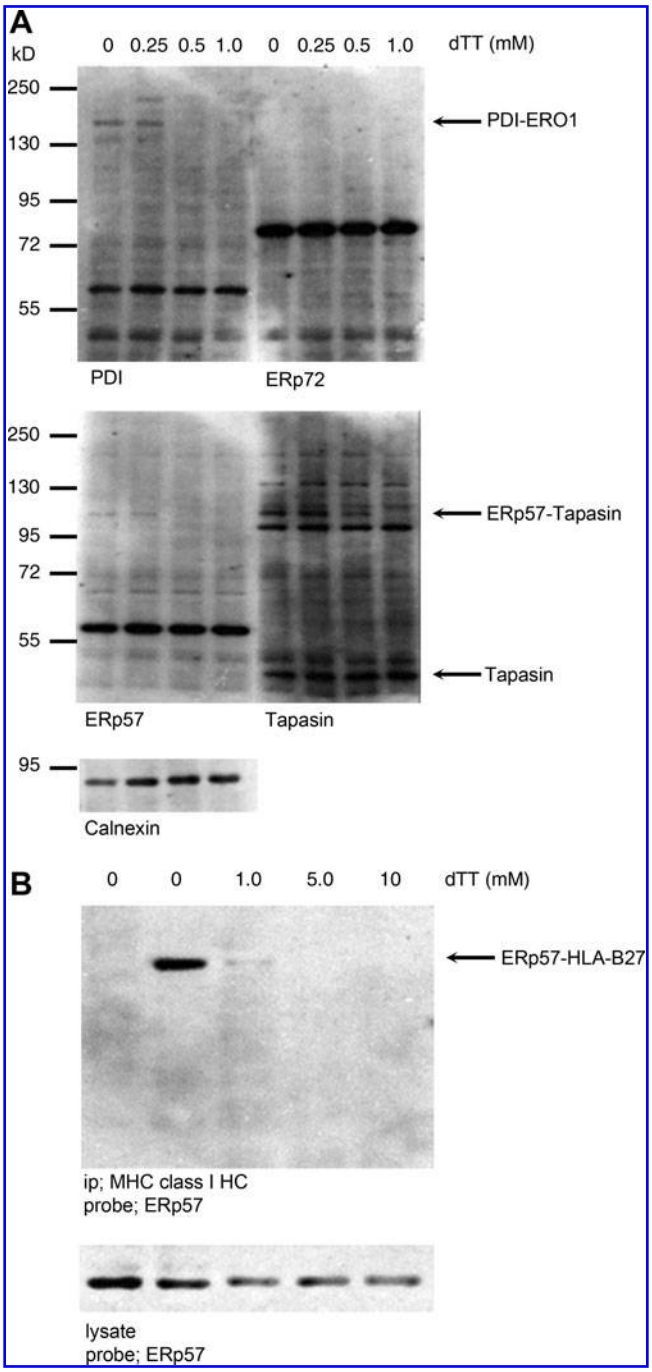

FIG. 5. High molecular weight forms of HLA-B27 do not appear to contain ERp57, ERp72, PDI, or tapasin. (A) Lysates generated from Figure 2C were analyzed by nonreducing SDS-PAGE and immunoblotted for PDI, ERp72, ERp57, and tapasin. Lysates were immunoblotted with CNX as a loading control. PDI-ERO1 and ERp57-tapasin conjugates are indicated by *arrows*. The tapasin monomer is indicated for clarity. **(B)** CEM.B27 cells were either untreated or incubated with 1.0, 5.0, and 10.0 m*M* dTT, NEM treated, lysed in 1% NP40 lysis buffer, and immunoprecipitated with pK antibody. Immunoprecipitates were analyzed by nonreducing SDS-PAGE and immunoblotted for ERp57, PDI (not shown), ERp72 (not shown), and tapasin (not shown). The ERp57-HLA-B27 conjugate is indicated by the *arrow*.

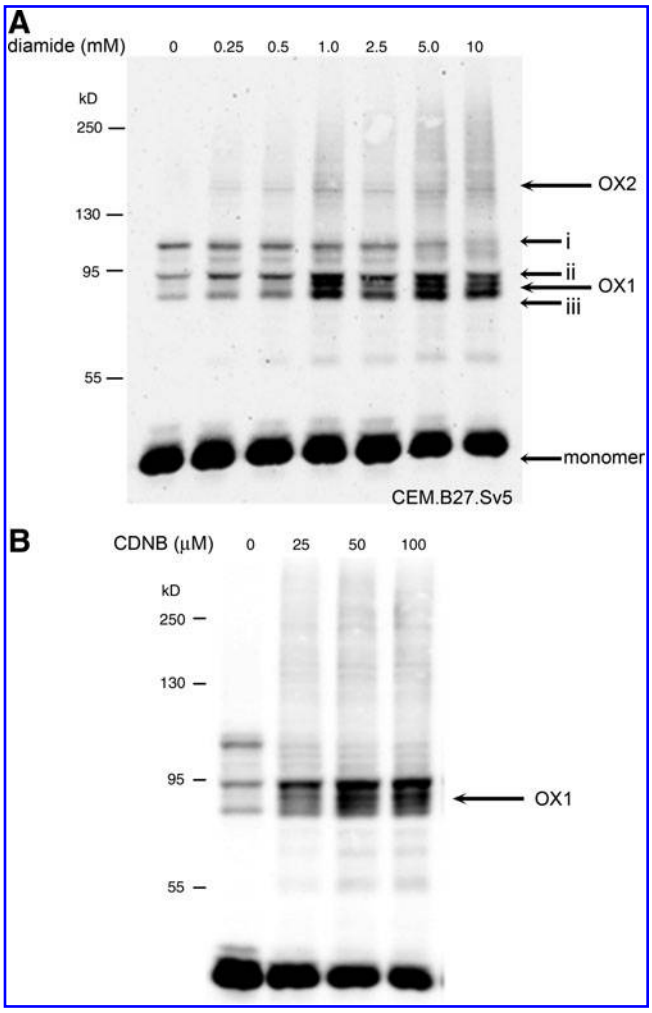

FIG. 6. HLA-B27 heavy chain dimers exhibit differential susceptibility to oxidative conditions. Equal numbers of CEM cells expressing HLA-B27 were incubated with increasing concentrations of the oxidizing agent **(A)** diamide (0, 0.25, 0.5, 1.0, and 10 m*M*) or CNDB (0, 25, 50, and 100 μM) and incubated at 37°C for 10 min (diamide) or **(B)** 1h (CNDB), NEM treated and lysed. Lysates were analyzed by nonreducing SDS-PAGE and immunoblotted with pK antibody. Heavy chain dimers are indicated by *arrows* i, ii, and iii. The band resulting from increasing oxidative conditions is indicated by *arrows* OX 1, whilst OX 2 indicates potential HLA-B27 aggregates. Monomeric heavy chain is indicated by *arrow* monomer.

susceptible to oxidation. The accumulation of bands that migrate with an apparent lower MW (Figs. 6A and B, OX1) and the resistance of band iii to increasing oxidation conditions suggested that these high MW species are composed of dimers with more 'compact' and/or oxidized heavy chains (Fig. 6).

Increasing levels of HLA-B27 heavy chain and heavy chain dimers lead to ER stress activation

Expression of HLA-B27 can lead to cellular stress in the form of the unfolded protein response (UPR) (26). The UPR is a cellular response to misfolding protein within the ER and

aims to restore ER homeostasis by enhancing folding or degradation of the misfolding protein (23, 29). Due to the potential adaptability of cells undergoing ER stress, we employed a transient expression system to determine whether HLA-B27 could indeed induce ER stress.

We chose to determine whether HLA-B27 could induce the activation of the ATF6 transcription factor by the generation of the cleaved cytosolic domain that is an early marker for ER stress (28). We transiently transfected HeLa cells with ATF6 FLAG tagged, followed by incubation with increasing concentrations of dTT to induce UPR. Lysates were generated and immunoblotted with an anti-FLAG antibody. With increasing dTT concentrations, we were able to detect increasing levels of the cleaved ATF6 cytosolic domain (Fig. 7A).

It has been reported that AS patients express higher levels of HLA-B27 (7) and that under physiological conditions such as IFN-γ induction, HLA-B27 can induce ER stress (25). Therefore, we wanted to determine whether increasing concentrations of HLA-B27 can lead to enhanced aggregation. HeLa cells were transduced with increasing amounts of lentivirus expressing HLA-B27. Increasing levels of HLA-B27 led to both enhanced cell surface expression and detection of HLA-B27 aggregates (Fig. 7B).

To determine whether increasing expression of HLA-B27 can lead to activation of ATF6, we co-transfected ATF6 and HLA-B27 into HeLa cells. We also co-transfected HLA-B7 and empty vector as negative controls. Immunoblotting and quantitation for the cleaved cytosolic domain demonstrated enhanced cleavage of the ATF6 transcription factor in the presence of increasing amounts of HLA-B27, when compared to the control tranfectants (Fig. 7C). FACS analysis of cells transduced with HLA-B27 and -B7 demonstrated equal levels of expression (data not shown). Thus increasing amount of HLA-B27 can induce aggregate formation and lead to the induction of the UPR.

Discussion

As part of our ongoing studies exploring the HLA-B27 dimerization process, we originally identified a role for the structurally conserved C164 residue (3). Here we provide further evidence for the role of C164. We originally proposed that the lack of dimerization was due to the absence of C164–C164 disulfide interactions (3). However, in the absence of both C164 and C67, dimerization was restored (Fig. 2B). Thus, when C164 is absent, C101 and C67 may participate in intramolecular interactions, which prevents and/or reduces their ability to form intermolecular disulfide bonds. In the absence of both C67 and C164, dimers probably occur via C101–C101 interactions, therefore suggesting both a novel role for C101 and C164 in HLA-B27 dimerization.

It remains undetermined whether HLA-B27 dimerization is a spontaneous event occurring in the oxidizing environment of the ER or is a chaperone/oxidoreductase-mediated event. Under certain physiological conditions, such as enhanced HLA-B27 production following maturation of DCs or in response to cytokines which upregulate MHC class I expression (21, 25), HLA-B27 exhibits an enhanced tendency to misfold and aggregate. Such dimerization may result from alterations in the oxidizing environment of the ER, which can accompany the maturation of dendritic cells in response to inflammatory stimuli. This latter scenario could be an important parameter in determining the role of HLA-B27 dimers during the development of inflammatory-associated arthritic disease.

The demonstration that HLA-B27 can enhance the activation of the UPR responsive ATF6 transcription factor (Fig. 7) suggests that cellular stress associated with misfolding HLA-B27 and/or aggregate formation could contribute to SpA pathology. This system models physiological settings which have been described in AS patients (7) and rat models of AS (14, 25, 26). The induction of ER stress by HLA-B27 has important implications for the proposed role of HLA-B27 in SpA development, as protein misfolding can induce proinflammatory cytokine production (30).

Using the cysteine mutants, a simplified model can be generated regarding the connectivity of C–C interactions of the different dimeric populations. The higher MW dimer (Fig. 2b, band F2) predominantly involves C164–C164 interactions, whilst lower MW dimeric populations require C101–C101 interactions as determined from the band patterns observed with the C67S–C101S and C67S–C164S mutants, respectively (Fig. 2). However, we believe this model too simplistic as it cannot explain why the C164S mutant prevents aggregation and why different redox conditions have such differential outcomes on dimeric conformations. We favor the idea that together with different cysteine pairings, there are accompanying redox differences in the heavy chain conformations and that HLA-B27 dimerization involves a preference or hierarchy of disulfide bonding. We propose that the predominant disulfide bond is mediated by C164–C164, followed by C67–C67 and C101–C101 interactions (Fig. 8). How these interactions are made is unknown, however, C67 and C101 will be the first translated cysteine residues within the ER lumen. Depending on the degree of co-translational folding, C67 and C101 could be in close proximity allowing the formation of an intramolecular disulfide bond. This would then leave C164 exposed and available to form intermolecular bonds. C101–C164 interactions may be restored, by isomerization reactions mediated by oxidoreductases such as ERp57 (2, 3), which may then allow subsequent C67–C67 interactions to occur. However, if C67 and C164 remain in inappropriate interactions this could leave C101 free to form non-native disulfide bonds (Fig. 8).

C101 and C164 could participate in dimerization in the absence of dramatic unfolding events as these residues have been proposed to be partially oxidized until MHC class I molecules reach the PLC (12). This could explain why multiple dimer species exhibit different electrophoretic mobility profiles. The dimers observed with the C67S–C101S and C67S–C164S mutants could be due to the interaction between two misfolded molecules, that would otherwise not occur with intact HLA-B27 molecules. However, where both C101 and C164 have been removed and presumably HLA-B27 is unfolded, we cannot detect any dimerization, suggesting HLA-B27 dimerization may be a more ordered event and not simply aggregation of misfolding molecules.

The differential susceptibility of the HLA-B27 dimer populations to reduction and oxidation suggests that these conformers exist in different redox states. Our study

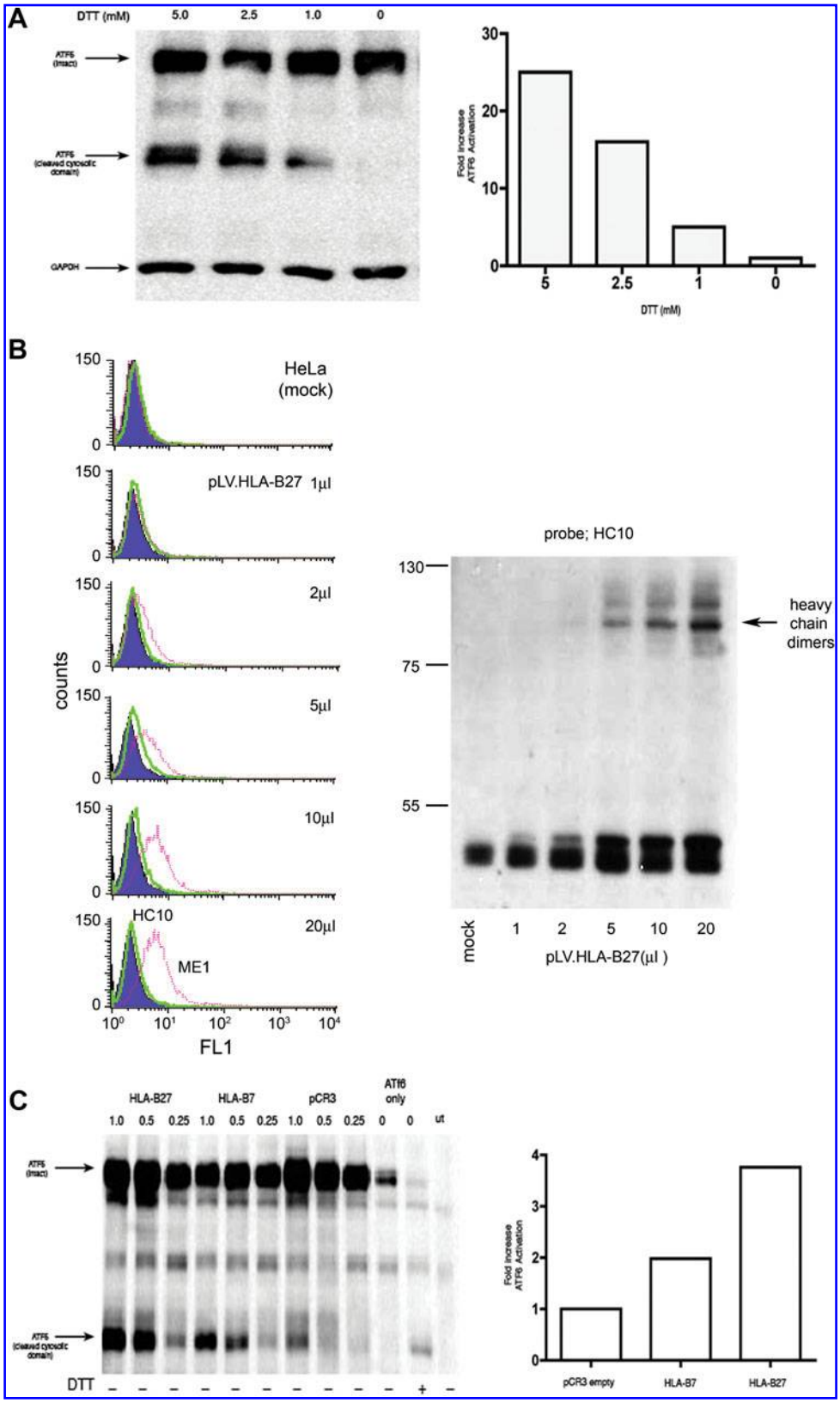

FIG. 7. HLA-B27 heavy chain dimers correlate with increased expression and processing of the UPR associated ATF6 transcription factor. (A) Equal numbers of HeLa cells were transiently transfected with ATF6-FLAG, followed by incubation with increasing concentrations of dTT (0, 1.0, 2.5, and 5.0 m*M*). Cells were lysed in nonreducing sample buffer and analyzed by immunoblotting with an anti-FLAG (for ATF6 activation) and GAPDH (loading control) antibodies. Cleaved cytolic domain ATF6 is indicated and quantitation of cleaved ATF6 is given in the *bar chart*. Quantitative measurements are given as a fold increase in ATF6 activation when compared to the transfected but non-ER stress induced control (0 m*M* dTT). **(B)** HeLa cells were transduced with increasing concentrations of HLA-B27 expressing lentivirus pLV.HLA-B27. Cells were analyzed for cell surface expression of HLA-B27 using ME1 and HC10 antibodies. Equal numbers of cells were lysed and immunoblotted with HC10 revealing increasing heavy chain dimers with increasing HLA-B27 expression. **(C)** HeLa cells were cotransfected with a constant amount of ATF6 (0.25 μg) and increasing concentrations of HLA-B27 or HLA-B7 or empty pCR3 vector (1.0, 0.5, and 0.25 μg). The amount of activated ATF6 was quantitated and the level of activation at 0.5 μg HLA-B27, -B, or empty vector is demonstrated. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

indicates that dimers are composed of multiple species, with each potentially exhibiting varying degrees of folding/unfolding. One possible explanation is that dimers with an apparent high MW represent two molecules in an unfolded conformation. This "open" conformation could dimerize via C101, C164, and C67 in varying orientations, accounting for an apparent enhanced resistance to dTT-mediated reduction. The susceptibility of the high MW conformer to diamide and CNDB suggests that the cysteines within this structure can be readily oxidized. The lower MW dimers

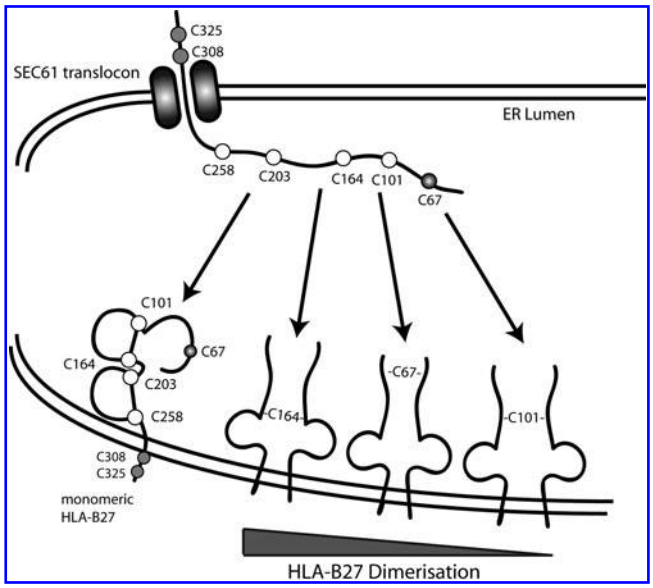

FIG. 8. Schematic demonstrating the preference or hierarchy of disulfide bonds used in HLA-B27 dimerization. C164–C164 is the dominant interaction, followed by C67–C67, and under certain conditions C101–C101 interactions can occur. However, mixed disulfides can occur, but for simplicity these have been excluded.

may represent heavy chains with a more folded conformation, with fewer C–C interactions. However, we cannot discount that some of these high MW species could represent conjugates between heavy chains and other unidentified proteins or oxidoreductases that are beyond the detection limits of our assays.

In conclusion, we have demonstrated that the conserved structural C101 and C164 residues are involved in HLA-B27 dimerization. HLA-B27 dimers are composed of multiple species with different redox states and/or conformations. These aberrant structures could well be involved in SpA development by triggering cellular stress responses. Our study therefore has important implications for determining the different conformational changes that can occur during the folding of HLA-B27 and the formation of associated dimer structures.

Materials and Methods

Cell lines and antibodies

C58, CEM, HOM2, and WEWAKI cell lines were maintained in RPMI 1640, supplemented with 10% FBS (Biosera, East Sussex, UK), penicillin, streptomycin and L-glutamine (R10 media) and maintained in a 5% CO_2 37°C incubator. HOM2 and WEWAKI cell lines were a kind gift from Dr. A. Benham (Durham University, UK). C58 cells expressing HLA-B*2705, HLA-B27.C67S, HLA-B27.C308S, HLA-B27.C325S, HLA-B27.C67S.C308S.C325S (cysteine triple mutant (TM)), HLA-B27.C101S, HLA-B27.C164S, HLA-B27.C101S–C164S, HLA-B27.C203S–C259S, HLA-B27.C67S–C101S, HLA-B27.C67S–C164S, HLA-B27.Δα3-domain and CEM cells expressing HLA-B*2705 were generated by electroporation at 900 μF, 180 V, and maintained in R10 media plus G418 (1 mg/ml) (Invitrogen, Paisley, UK). Anti-V5 (pK) and anti-flag tag antibodies were obtained from Serotec and Stratagene (La Jolla, CA), respectively. Monoclonal antibody HC10 (a kind gift from Prof. T. Elliott, Southampton University, UK). recognizes unfolded HLA-B and -C molecules (24) and ME1 conformational specific antibody recognizes fully folded HLA-B27. Anti-CNX, -ERp72, and -PDI antibodies were obtained from AbD Stressgen (Oxford, UK) and anti-ERp57 from Abcam (Abcam, Cambridge, UK). Anti-tapasin rabbit antisera was a kind gift from Prof T. Elliott. Goat anti-mouse HRP conjugated secondary antibody was obtained from Caltag Laboratories (Buckingham, UK) and anti-rabbit monoclonal HRP from Sigma (Poole, Dorset, UK).

Monocyte-derived dendritic cells

AS patient monocyte-derived dendritic cells (DC) were obtained under written consent and ethical review by NHS National Ethics Service and local School Of Medicine ethics committee. The plastic adherent population of blood monocytes were cultured in 50 ng/ml IL-4 and 50 ng/ml GM-CSF (R and D Systems, Abingdon, UK) for 5 days, before treatment with 100 ng/ml lipopolysaccharide (LPS) (Sigma).

Site-directed mutagenesis

A V5 C-terminally tagged cDNA encoding the HLA-B*2705 allele, cloned into the mammalian expression vector pCR3.1 (Invitrogen) was mutated using PCR-based site directed mutagenesis (Quickchange, Stratagene, La Jolla, CA) according to manufacturers instructions (2). All constructs were verified by sequencing (Dundee University, Sequencing Service, UK).

NEM immunoprecipitation

Approximately 5×10^7 cells were incubated with 20 m*M* NEM (Sigma)/PBS pH 7.0 on ice for 20 min, then lysed in 1% NP40 lysis buffer supplemented with 1 m*M* PMSF (Sigma), 1x complete protease inhibitors (Roche, Germany), and 10 m*M* NEM. Lysates were precleared for 1 h at 4°C, and immunoprecipitated with either pK or HC10 antibody, 2 h at 4°C. Immunoprecipitates were washed with 1% NP40 buffer.

Heavy chain dimer detection

Equal numbers of cells were pretreated with 20 m*M* NEM/PBS pH 7.0 before lysis in 1% NP40 lysis buffer. Lysates were separated on 8% SDS PAGE and immunoblotted either with pK and HC10 antibodies. To determine the effects of a reducing or oxidizing environment on heavy chain dimers, the CEM cell line expressing HLA-B27 were incubated for 10 min at 37°C with either increasing concentrations of dTT (Fluka-Sigma) or diamide (Sigma), respectively, followed by NEM treatment and lysis in 1% NP40 and immunoblotting. To determine the contribution of noncovalent associations in HLA-B27 heavy chain dimer formation, lysates were incubated with 8 *M* urea at increasing temperatures prior to immunoblotting analysis.

Immunoblotting

Samples were resolved on 8% SDS-PAGE, transferred onto nitrocellulose (BA85, Whatmann, UK), blocked for 1 h with 5% skimmed milk powder in PBS/0.1% Tween (Sigma), followed by overnight incubation with respective primary antibodies. Images were detected by chemiluminescence using Supersignal Femto (Pierce, Illinois).

Flow cytometry

Cells were resuspended and fixed in 3.8% paraformaldehyde (PFA)/PBS for 10 min and stained with ME1 supernatant (50 μl) or HC10 (20 μg/ml) for 60 min at 4°C, washed 3X with PBS/1% FBS (FACS buffer), followed by incubation with anti-mouse FITC conjugated antibody (Sigma). Samples were washed 3X, analyzed on FACS Calibre flow cytometer and acquired using FlowJo software (Becton and Dickenson). For intracellular pK staining, cells were fixed as above, followed by permeabilization with 1X FACSPerm buffer (BD Biosciences). Permeabilized samples were incubated with anti-pK antibody (1 mg/ml) overnight at 4°C, washed with FACS buffer, followed by incubation for 2 h at 4°C with anti-mouse FITC conjugated antibody and acquired on FACS Calibre.

Transient transfections and ER stress assays

HeLa cells at 5×10^5 per well were transduced with pDUAL.GFP lentivirus expressing HLA-B27. 18 h later cells were harvested for FACS analysis and immunoblotting. ER stress assays; HeLa cells were co-transfected with 0.25 μg ATF6-FLAG and increasing concentrations of HLA-B27, -B7, or pCR3 plasmid using PEI transfection reagent according to manufacturer's conditions. 18 h later cells were lysed in SDS sample buffer. Lentiviruses expressing HLA-B*2705 were generated by transient co-transfection of 1×10^7 293T cells with 3.75 μg B27-lentiviral vector, 2.5 μg of pCMV_R8.9 envelope plasmid, and 2.5 μg of the vesicular stomatitis virus glycoprotein (VSV.G)-encoding plasmid using FuGENE (Roche). Supernatants were collected and filtered 72 h post transfection.

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Author Disclosure Statement

The authors would like to state that no competing financial interests exist

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Abbreviations Used

β2m = beta 2-microglobulin
BiP = immunoglobulin binding protein
C = cysteine
CNDB = 1-chloro-2,4-dinitrobenzene
CNX = calnexin
DC = dendritic cell
dTT = dithiothreitol
EBV = Epstein Barr virus
ECL = enhanced chemiluminescence
endoH = endoglycosidase H
ER = endoplasmic reticulum
FBS = fetal bovine serum
GSH = reduced glutathione
HLA = human leukocyte antigen
LIR = leukocyte inhibitory receptor
MFI = mean fluorescence intensity
MHC = major histocompatibility complex
mM = millimolar
LPS = lipopolysaccharide
MW = molecular weight
NEM = N-ethyl-malemide
NK = natural killer
PBS = phosphate buffered saline
PDI = protein disulfide isomerase
PFA = paraformaldehyde
PLC = peptide loading complex
S = serine
SDS = sodium dodecyl sulfate
SpA = spondyloarthropathies
TAP = transporter associated with antigen processing
TM = triple mutant

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