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Differential Oxidation of HLA-B2704 and HLA-B2705 in Lymphoblastoid and Transfected Adherent Cells

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ABSTRACT

MHC class I molecules are predominantly involved in the presentation of antigens from viral proteins to CD8+ T cells of the immune system. However, MHC proteins can also be linked to autoimmune diseases, and the HLA-B27 allele is expressed by 95% of people with the rheumatic condition ankylosing spondylitis (AS). A precise molecular explanation for the association between HLA-B27 and AS is still lacking, although it is known that inappropriately disulfide bonded HLA-B27 heavy chains can be found at both the cell surface and in the endoplasmic reticulum (ER) of HLA-B27 expressing cells. This papers shows that HLA-B27 heavy chain misfolding does not depend on any unpaired cysteine residue *per se* when HLA-B27 is highly expressed. Also shown is that major differences exist in the disulfide-dependent conformations of two HLA-B27 subtypes, HLA-B2704 and HLA-B2705. The results imply that residues 77, 152, and/or 211 influence the redox potential of the MHC class I heavy chain and suggest that manipulating the redox environment can alter the conformational state of HLA-B27 subtypes. *Antioxid. Redox Signal.* 8, 292–299.

INTRODUCTION

MHC CLASS I HEAVY chains assemble with the light chain, beta2microglobulin (β2m), and an antigenic peptide of around 9 amino acids in the endoplasmic reticulum (ER), prior to transport to the cell surface. The assembly process is overseen by a number of accessory proteins and chaperones including the antigen transporter TAP, the bridging molecule tapasin, the chaperones calnexin and calreticulin, and the oxidoreductase ERp57 (8). Two intramolecular immunoglobulin-like disulfide bonds are formed in MHC class I heavy chains relatively early in the folding process (21). Correctly folded class I molecules are exported from the ER, whereas misfolded molecules are likely to be expelled from the compartment for degradation in the cytosol (12). The control of MHC class I heavy chain oxidation is a particularly important issue with regard to ankylosing spondylitis (20). This autoimmune rheumatic condition (and the related Reiters disease), mainly affects young men, and results in characteristic deformity and fusion of the sacroiliac joints. The HLA-B27 MHC class I allele is expressed by 95% (but not all) of sufferers, making this genetic association one of the strongest known. HLA-B27 distributions are highest in Amerindian (5%) and Australian aboriginal (6%) populations.

Although the precise role of HLA-B27 in AS is not completely understood, it is known that HLA-B27 can form heavy chain-heavy chain (HC-HC) homodimers instead of HC-β2m heterodimers (1). These HC-HC homodimers and other HLA-B27 structures have the potential to be differentially recognized by various immune cell receptors (2, 16). Molecular mimicry has also been implicated in AS, since increased load of *Klebsiella aerogenes* and other microorganisms is often seen in sufferers and some naturally occurring HLA-B27 peptides are very similar to bacterial antigens presented by HLA-B27 (19). HC–HC homodimers can form both in the ER and at the cell surface (7), where the conversion of HC-β2m to homodimer is likely to lead to the loss and subsequent

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deposition of β2m in the joints and capillaries (28). HLA-B27 has an unusual cysteine residue at position 67, located on an alpha helix close to the peptide-binding site. C67 has been implicated in the formation of HC-HC dimers because of the potential of this unpaired cysteine to form a disulfide bond (5). However, the C67 residue is present in almost all subtypes of HLA-B27 (except B2718 and B2723).

The HLA-B2705 subtype is strongly linked to AS, and as a result HLA-B2705 has been the most intensively studied allele with respect to rheumatic disease. However, the biochemistry of other AS-associated HLA-B27 alleles, such as HLA-B2704, has been less well documented. Despite the close association between AS and HLA-B27 expression, not every HLA-B27 allele is closely linked to the disease. HLA-B2706 and HLA-B2709, for example, do not appear to predispose carriers to AS (11), even though they differ from HLA-B2705 by only one amino acid residue (position 116 for HLA-B2709) or five amino acid residues (position 74, 114, 116, 152, 211 for HLA-B2706). AS can also occur in a minority of cases without a C67-containing allelic product being present.

Although the role of C67 in HLA-B2705 HC-HC dimers has been well studied (3, 4, 7, 9), HLA-B2705 also contains two other unpaired cysteines, C308 and C325, in addition to its two disulfide bridges. The potential involvement of C308 and C325 in HC-HC dimerization has not been documented, probably because these two cysteines are in or close to the transmembrane sequence. However, transmembrane disulfides have been found in some proteins (e.g., the Golgi enzyme fucosyltransferase III) (22). Although disulfides do not readily form in cytosolic proteins, this can occur in times of oxidative stress (17). In this paper, we investigate the oxidation of HLA-B2705 by examining the role of the three unpaired cysteine residues, and by comparing the oxidation of HLA-B2704 to HLA-B2705 in immortalized lymphoblastoid cells. We find that high expression of all HLA-B2705 mutants tested results in oxidative misfolding and BiP binding, but that in lymphoblastoid cell lines, HLA-B2705 and HLA-B2704 have different reactivities towards W6/32, a conformation specific MHC class I antibody. Furthermore, the conformation of HLA-B proteins can be manipulated in living cells using a chemical redox reagent, the Protein Disulfide Isomerase (PDI) mimic BMC.

MATERIALS AND METHODS

Chemicals

All chemicals were from Sigma (Poole, Dorset, UK) unless otherwise stated. BMC [*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane] (29) was a kind gift from R. Raines and was made fresh as a stock solution of 100 m*M* in DMSO.

Cell lines and DNA constructs

The human fibrosarcoma cell line HT1080 was maintained in Dulbecco's Modified Eagle's medium (DMEM). Cells were supplemented with 8% fetal calf serum (FCS) (Sigma), 2 m*M* glutamax, 100 units/ml penicillin, and100 g/ml streptomycin (Invitrogen, Paisley, UK). The human lymphoblastoid cell lines BTB, BRUG, JESTHOM, HOM-2 (all HLA-B2705), and WEWAK1 (HLA-B2704) were obtained from the ECACC (European collection of cell cultures). The DW cell line (HLA-B2704) was a kind gift from P. Bowness (University of Oxford, UK). These cell lines were cultured in RPMI 1640 supplemented with 8% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin and 2 m*M* glutamax. The cDNA encoding wild-type HLA-B2705 was a gift from J. Neefjes (Netherlands Cancer Institute, Amsterdam, Netherlands). The mutants C67A, C308A and C325A were made by site directed mutagenesis (Quickchange, Stratagene, La Jolla, CA, USA). All pcDNA3 constructs were confirmed by DNA sequencing. Numbering of the MHC class 1 heavy chain starts from the first amino acid after signal peptide cleavage.

Antibodies

The conformation-specific monoclonal antibody (mAb) W6/32 (18) and the H chain-specific mAb HC10, recognizing free B and C allele heavy chains (23), were gifts from J. Neefjes. The polyclonal rabbit anti-serum against PDI has been described (6). Polyclonal antisera against BiP/grp78 (antibody N-20) and human β2m (antibody FL-119) were both from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A second β2m antiserum raised against the entire polypeptide was obtained from Sigma.

Transfections

Transfections were carried out with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Subconfluent cells in 6 cm dishes were washed twice with PBS (Invitrogen), and transfected with 1 or 2 μg DNA for 6 h in the presence of Optimem serum free medium (Invitrogen). The medium was replaced after 6 h with complete medium and the cells analyzed 24 h post-transfection.

Immunoblotting

Cell lysates were prepared in 100 m*M* NaCl, 30 m*M* Tris, 20 m*M* MES, pH 7.4, with 1% Triton X-100 and 10 μg/ml of protease inhibitors chymostatin, leupeptin, antipain, and pepstatin A (Sigma). Alkylation was with 20 m*M* *N*-ethylmaleimide (NEM) in the lysis buffer where required. Nuclei were removed by centrifugation at 16,100 *g* for 10 min at 4°C. The lysates were taken up into 2× Laemmli buffer and either heated for 4 min at 95°C or left at room temperature. Postnuclear cell lysates were analyzed by 10% SDS-PAGE under both reducing (50 m*M* DTT) and nonreducing conditions. Proteins were blotted onto PVDF membranes (Millipore, Watford, Hertfordshire, UK) at 150 mA for 2 h or 30 V overnight. The membranes were blocked in 8% powdered milk in PBS plus 0.1% Tween-20 (PBST) for 1 h or overnight and then the membranes were probed with relevant antibodies. The primary antibodies were used at 1:250 (W6/32 and HC10 tissue culture supernatant), 1:1000 (anti-PDI, anti-β2m), and 1:200 (anti-BiP) for 1 h. After four washes with PBST, membranes were incubated with the corresponding secondary antibody (DAKO, Ely, Cambridgeshire, UK) at 1:1000 (anti-mouse) and 1:3000 (anti-rabbit) for 1 h, washed extensively, and visualized by ECL (Amersham, GE Healthcare, Pollards Wood Buckinghamshire, UK) and exposure to

film (Kodak Biomax film, Sigma). Protein markers were from Bio-Rad (Hemel Hempstead, Hertfordshire, UK).

Immunoisolations

Cells were lysed as described above. Immunoisolations were performed using 50 µl of HC10 supernatant or 4 µl of β2m antisera immobilized on 50 µl of a 20% suspension of Protein A sepharose beads for 1 h at 4°C. Pellets were washed twice with 100 m*M* NaCl, 30 m*M* Tris, 20 m*M* MES, 1% Triton X-100, pH 7.4, and taken up in sample buffer. Analysis was by 10% SDS-PAGE and immunoblotting as described above.

RESULTS

Oxidative misfolding of HLA-B27 is C67, C308 and C325 independent

In order to address the role of all free cysteine residues in HLA-B2705 heavy chain misfolding and oxidation, we created three cysteine mutants from wtHLA-B2705: C67A, C308A, and C325A. Wild-type HLA-B2705 and the C67A mutant were transfected into HT1080 cells. The cells were lysed in the presence of 20 m*M* NEM. Samples were either boiled for 4 min or left at room temperature before analysis by nonreducing and reducing SDS-PAGE, followed by blotting with the MHC class I heavy chain specific Mab HC10. Figure 1A shows that both the wild-type and C67A mutant formed extensive disulfide-dependent HC complexes, the majority of which were dispersed upon reduction of the samples with DTT (Fig. 1B). The extent of intermolecular disulfide bond formation did not depend on whether the samples were boiled (Fig. 1A, compare lane 1 with 2). A major HC10 reactive, DTT resistant band could clearly be seen at 80–90 kD in the reduced samples from the transfected cells (Fig. 1B). This band was partly sensitive to boiling (compare Fig. 1B, lanes 1 and 3 to lanes 2 and 4) and appeared in both wild-type and mutant transfectants. The appearance of this band was reproducible but its intensity varied somewhat between experiments and was dependent on transfection efficiency (data not shown).

Given that the disulfide-dependent HC complexes were not dependent on the C67 residue, we tested whether C308 and C325 were involved in their formation. Thus HT1080 cells were transfected with the HLA-B2705 C67A, C308A, and C325A mutants and the boiled samples were analyzed as before by nonreducing (Fig. 1C) and reducing (Fig. 1D) SDS-PAGE. All three cysteine mutants clearly formed extensive disulfide dependent complexes (Fig. 1C, lanes 1–3) that behaved similarly with or without boiling of the samples (not shown). These disulfide-dependent complexes could be mostly disrupted by reduction (Fig. 1D, lanes 1–3), but each mutant class I molecule could be found in DTT and SDS resistant complexes (Fig. 1D, lanes 1–3). The appearance of the doublet varied between experiments, and similar results were obtained using both HT1080 and HeLa cell lines (not shown).

The DTT resistant bands seen in Figure 1 could be residual HC-HC dimers, or HC in association with BiP/grp78, an interaction that remains stable under reducing conditions (see

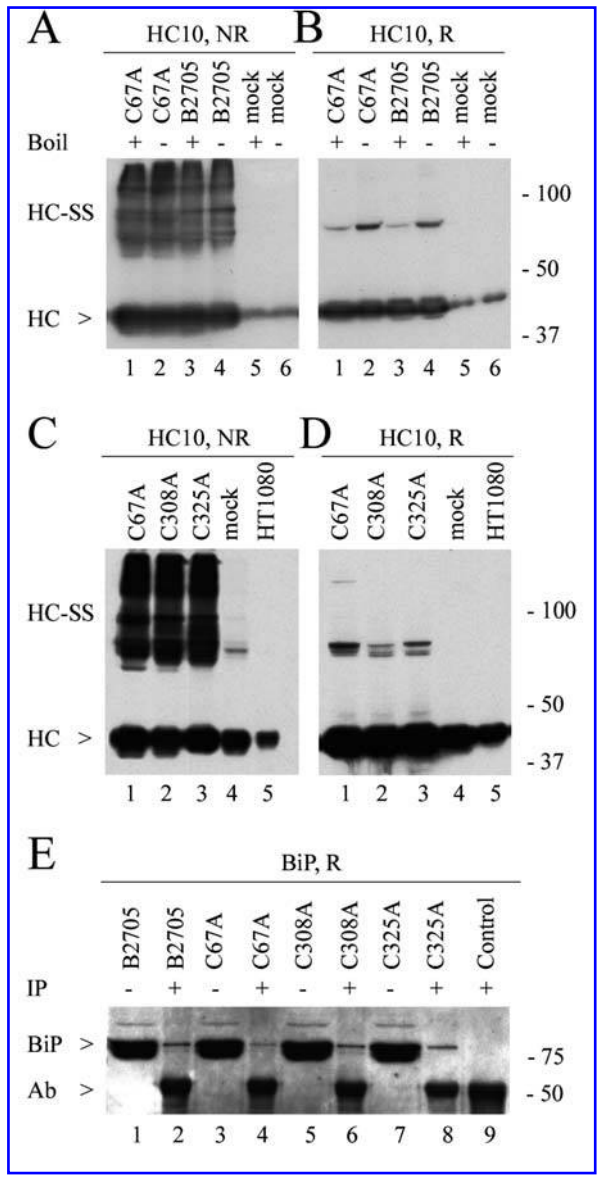

FIG. 1. Oxidative misfolding of HLA-B27 MHC class I heavy chains in HT1080 cells. (**A**) Lysates from HT1080 cells transfected with HLA-B2705 C67A (*lanes 1 and 2*), HLA-B2705 wild type (*lanes 3 and 4*) or mock (*lanes 5 and 6*) were analyzed by nonreducing SDS-PAGE and immunoblotted with HC10 with (*lanes 1, 3, 5*) or without (*lanes 2, 4, 6*) boiling. Monomeric MHC class I molecules (HC) and disulfide-linked complexes (HC-SS) are indicated. (**B**) As for (**A**) except samples analyzed under reducing conditions. (**C**) Lysates from HT1080 cells transfected with HLA-B2705 C67A (*lane 1*), HLA-B2705 C308A (*lane 2*), HLA-B2705 C325A (*lane 3*), mock (*lane 4*), or untransfected (*lane 5*) were analyzed by nonreducing SDS-PAGE and immunoblotted with HC10. Monomeric MHC class I molecules (HC) and disulfide-linked complexes (HC-SS) are indicated. (**D**) As for (**C**) except samples analyzed under reducing conditions. (**E**) Lysates (*lanes 1, 3, 5, 7*) and HC10 immunoprecipitations (*lanes 2, 4, 6, 8*) from HT1080 cells transfected with HLA-B2705 (*lanes 1 and 2*), HLA-B2705 C67A (*lanes 3 and 4*), HLA-B2705 C308A (*lanes 5 and 6*), HLA-B2705 C325A (*lanes 7 and 8*), and mock (*lane 9*) were subjected to reducing SDS-PAGE and immunoblotted for BiP.

Fig. 1B, Ref. 4 and Ref. 27). To determine whether BiP associated with HLA-B2705 and the three cysteine mutants, HT1080 cells were transfected with these constructs and lysed in the presence of 20 m*M* NEM. The cell lysates were investigated directly for the presence of BiP (Fig. 1E, lanes 1, 3, 5, and 7) or were subjected to immunoprecipitation with HC10 prior to blotting the immunoprecipitates with αBiP (Fig. 1E, lanes 2, 4, 6, and 8). BiP could be co-immunoprecipitated with wild-type HLA-B2705 (Fig. 1E, lane 2) and with C67A, C308A, and C325A (Fig. 1E, lanes 4, 6, and 8). A non-transfected control showed that BiP did not co-immunoprecipitate with antibody (Fig. 1E, lane 9). From these experiments, we conclude that both oxidative misfolding of HLA-B2705 heavy chains and BiP association is independent of C67A, C308A, and C325A in transfected cell lines.

HLA-B2704 and HLA-B2705 have different disulfide-dependent conformations

Since high expression levels of HLA-B2705 result in oxidative misfolding in transfected cells, we turned to HLA-typed lymphoblastoid cell lines to examine the behavior of HLA-B27 alleles that are associated with AS. It was previously reported that HLA-B2705 exhibits an unusual reactivity towards the conformation-specific antibody W6/32. W6/32 normally recognizes peptide loaded MHC class I molecules (18), but it has been shown to recognize some B allele proteins when immunoblotting nonreducing gels (26). We tested the reactivity of W6/32 towards HLA-B2705 (from the cell line BTB) and HLA-B2704 (from the cell line WEWAK1) (Fig. 2). Cells were lysed in the presence of NEM and the samples were subjected to boiling for 4 min (Fig. 2A, lanes 1 and 3) or left at room temperature (Fig. 2A, lanes 2 and 4). A population of HLA-B2705 molecules reacted with W6/32 when the samples were not boiled (Fig. 2A, lane 2), and the reactivity disappeared upon boiling (Fig. 2A, lane 1), consistent with the findings of Tran et al. (26). The disappearance of the W6/32 signal upon boiling is not related to overall loss of MHC class I molecules upon boiling or reduction (see Figs 1 and 2B, and data not shown).

Unexpectedly, HLA-B2704 molecules behaved differently from HLA-B2705 molecules. The W6/32-reactive HLA-B2704 population was independent of boiling and migrated faster than the HLA-B2705 W6/32-reactive pool (Fig. 2A, lanes 3 and 4). To confirm migration and equal loading of samples, the membranes were also probed using a polyclonal serum raised against the ER protein PDI (Fig. 2A, lanes 1–4). No signal could be detected with W6/32 when proteins were run under reducing conditions, as expected (not shown). The HC10 reactive pool of MHC class I molecules expressed by WEWAK1 and BTB cells was indistinguishable (Fig. 2B, lanes 1–4).

To test whether our observations were true of HLA-B2704/05 alleles in general, we examined other HLA typed lymphoblastoid cell lines. HLA-B2705 expressing BRUG cells and HLA-B2704 expressing DW cells were lysed in the presence of NEM, and the nonreduced MHC class I proteins were analyzed for their reactivity towards W6/32. Figure 2C shows that HLA-B2705 W6/32-reactive proteins from BRUG migrated more slowly than their counterparts from DW cells,

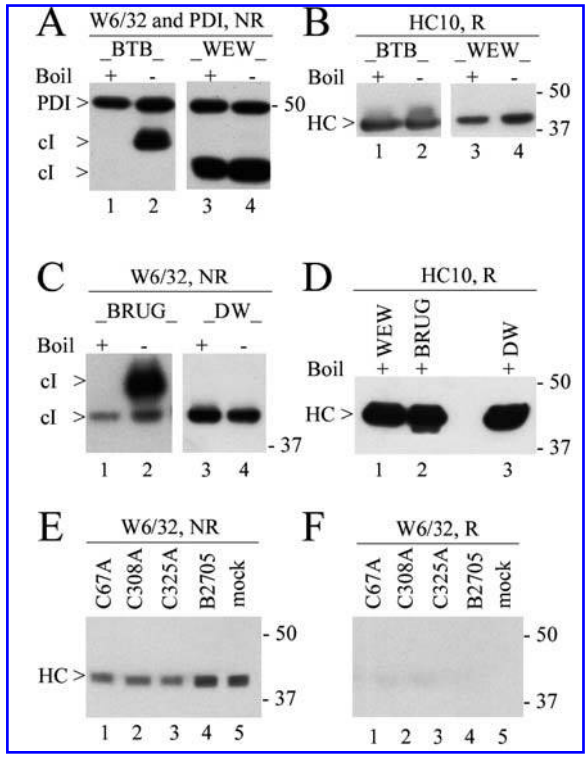

FIG. 2. Allele specific differences in the conformation of HLA-B27. (**A**) Lysates from BTB cells (HLA-B2705; *lanes 1 and 2*) and WEWAK1 cells (HLA-B2704; *lanes 3 and 4*) were subjected to nonreducing SDS-PAGE with (*lanes 1 and 3*) or without (*lanes 2 and 4*) boiling. Immunoblotting was with W6/32 and PDI antisera. cI = MHC class I. (**B**) Lysates from BTB cells (*lanes 1 and 2*) and WEWAK1 cells (*lanes 3 and 4*) were subjected to reducing SDS-PAGE with (*lanes 1 and 3*) or without (*lanes 2 and 4*) boiling. Immunoblotting was with HC10. (**C**) Lysates from BRUG cells (HLA-B2705; *lanes 1 and 2*) and DW cells (HLA-B2704; *lanes 3 and 4*) were subjected to nonreducing SDS-PAGE with (*lanes 1 and 3*) or without (*lanes 2 and 4*) boiling. Immunoblotting was with W6/32. cI = MHC class I. (**D**) Lysates from WEWAK1 (*lane 1*), BRUG (*lane 2*), and DW cells (*lane 3*) were subjected to reducing SDS-PAGE and immunoblotting with HC10. (**E**) Lysates from cells transfected with HLA-B2705 C67A (*lane 1*), HLA-B2705 C308A (*lane 2*), HLA-B2705 C325A (*lane 3*), wt (*lane 4*), and mock (*lane 5*) were analyzed by nonreducing SDS-PAGE and immunoblotted with W6/32. Monomeric MHC class I molecules (HC) are indicated. (**F**) As for (**E**) except samples analyzed under reducing conditions.

and that the W6/32-reactive population from DW was resistant to boiling. No differences between DW and BRUG could be seen in the HC10 reactive pool of proteins (Fig. 2D). Another HLA-B2704 expressing cell line, DH, exhibited a similar pattern to DW and WEWAK1 (not shown). We therefore conclude that HLA-B2704 molecules do not form slow migrating temperature-sensitive W6/32-reactive structures. Thus major differences exist between the conformation of W6/32-reactive HLA-B2704 and HLA-B2705 molecules in human lymphoblastoid cell lines.

Next, we asked whether HLA-B2705 molecules in transfected cells behaved the same as endogenously expressed HLA-B2705 molecules. Thus the lysates from cells transfected with HLA-B2705 and the three HLA-B2705 cysteine mutants (shown in Fig. 1) were subjected to Western blotting and the membranes probed with W6/32, after nonreducing (Fig. 2E) and reducing (Fig. 2F) SDS-PAGE. No characteristic slow-migrating HLA-B2705 W6/32 reactive band was seen in the nonreducing gels (Fig. 2E, lanes 1–4) despite the fact that endogenous W6/32 reactive HLA-B proteins were detected under these conditions. This result suggests that W6/32 does not recognize misfolded MHC class I heavy chains. Interestingly, cells co-transfected with human β2m and HLA-B2705 do exhibit nonreducing "slow migrating" W6/32 reactivity (26), suggesting that W6/32 recognizes a nonreduced, SDS-resistant complex between HLA-B2705 and β2m. To investigate the role of β2m further, we analyzed the HLA-B2704 expressing cell line WEWAK1 and two HLA-B2705 expressing cell lines, JESTHOM and HOM-2. Lysates from these cell lines were analyzed by Western blotting using the β2m specific antisera FL-119 after both reducing (Fig. 3A) and nonreducing (Fig. 3B) SDS-PAGE. Although a monomeric β2m signal could clearly be detected in the reducing gel (Fig. 3A), no signal could be detected in the nonreducing gels in the region where HC-β2m complexes should migrate, regardless of whether the samples were boiled prior to electrophoresis (Fig. 3B). Similar results were obtained with a different β2m antiserum raised against the whole sequence (not shown). The presence of W6/32 reactive molecules under nonreducing conditions was confirmed by probing the membranes with W6/32 (Fig. 3C). Both JESTHOM (Fig. 3C, lanes 3–4, cI*) and HOM-2 (Fig. 3C, lanes 5–6, cI*) had temperature-dependent, W6/32 reactive HLA-B2705 molecules as expected.

The absence of a β2m signal in the 30–50 kD region of the nonreducing gels suggested that β2m was absent from the W6/32-reactive band. However, this result could be explained by a lack of reactivity to buried or lost epitopes. We complemented this experiment by asking whether properly folded HC-β2m complexes contained material that could be recognized by W6/32 after nonreducing SDS-PAGE. To do this, we used the β2m antiserum (raised against the full sequence) to immunoprecipitate β2m and β2m-HC complexes from WEWAK1, JESTHOM, and HOM-2 cells. This was followed by blotting the nonreduced immunoisolates with W6/32 (Fig. 3D). As a control, β2m was successfully immunoisolated from each lysate and detectable under reducing conditions (Fig. 3D, lanes 1–3). W6/32 reactive complexes could be detected strongly in immunoisolates from WEWAK1 cells (Fig. 3D, lane 5) and very weakly in immunoisolates from JESTHOM and HOM-2 cells (lanes 2 and 3) under nonreducing conditions. Note that HLA-B2705 molecules could not be assessed, because boiling was required to visualize the immunoisolated material. This experiment suggested that "nonreducing" W6/32 reactive HLA–B2704 heavy chains are assembly-competent, not misfolded, and are capable of forming complexes with β2m. Although previous investigators have noted the presence of β2m in W6/32-reactive, nonreducing HLA-B2705 complexes (26), our experiments cannot definitively prove whether β2m was present or absent from all HLA-B27 SDS-resistant complexes.

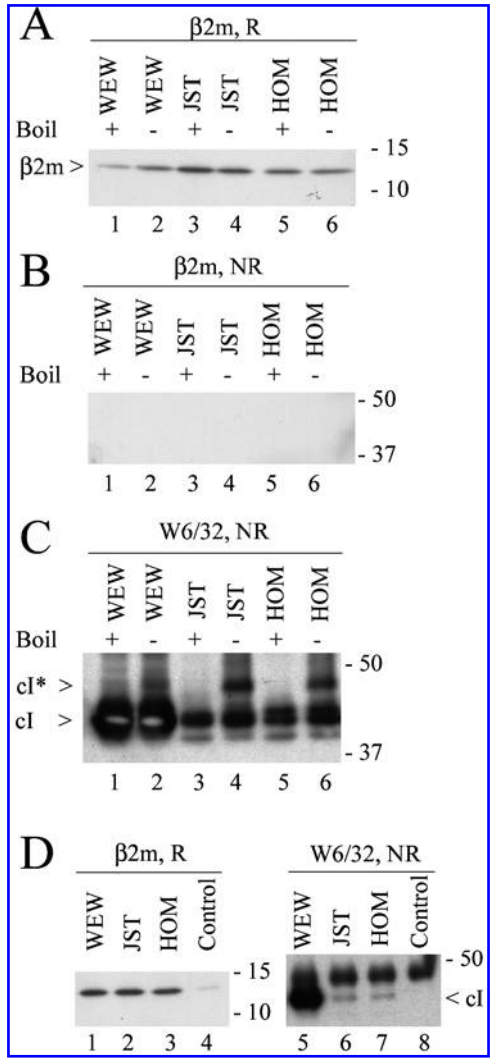

FIG. 3. β2m reactivity in lymphoblastoid cell lines. (**A**) Lysates from WEWAK1 (HLA B2704; WEW, *lanes 1 and 2*), JESTHOM (HLA-B2705; JST, *lanes 3 and 4*), and HOM-2 (HLA-B2705; HOM, *lanes 5 and 6*) cells were analyzed by reducing SDS-PAGE and immunoblotted with β2m serum with (*lanes 1, 3, 5*) or without (*lanes 2, 4, 6*) boiling the samples for 4 min prior to electrophoresis. (**B**) As for (**A**) except samples analyzed under reducing conditions. (**C**) As for (**A**) except samples analyzed by immunoblotting with W6/23 after nonreducing electrophoresis. (**D**) Lysates from WEWAK1, JESTHOM, and HOM-2 cells were subjected to immunoisolation with β2m serum, and the immunoprecipitates were analyzed by SDS-PAGE prior to immunoblotting with β2m serum FL-119 (*lanes 1–3*) or with W6/32 (*lanes 5–7*). The control is antibody alone (*lanes 4 and 8*). cI* = temperature sensitive HLA-B2705.

The W6/32 reactive pool of HLA-B molecules is redox sensitive

The results in Figure 2 suggested that the differences in conformation between HLA-B2704 and HLA-B2705 were

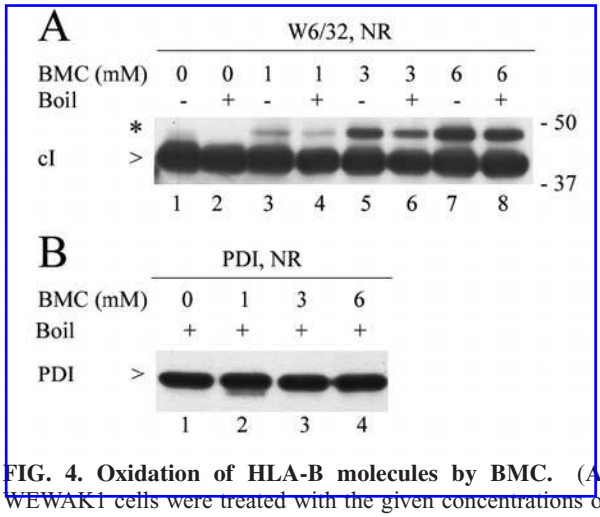

FIG. 4. Oxidation of HLA-B molecules by BMC. (**A**) WEWAK1 cells were treated with the given concentrations of BMC for 3 h. Cell lysates were analyzed by nonreducing SDS-PAGE with (*lanes 2, 4, 6, 8*) and without (*lanes 1, 3, 5, 7*) boiling the samples and immunoblotted using W6/32. cI = MHC class I. * = BMC induced, W6/32 reactive conformer. (**B**) The lysates from (**A**) were subjected to SDS-PAGE and immunoblotted using PDI antiserum.

disulfide dependent, since reducing the samples with DTT destroyed the W6/32 epitope. We therefore tested whether changing the redox conditions could influence the conformation of HLA-B proteins. To do this, we used BMC, a redox reagent that has been described as a peptide mimic of Protein Disulfide Isomerase (PDI). PDI can catalyze the oxidative folding of proteins in the ER (29). WEWAK1 cells were incubated with increasing concentrations of BMC for 3 h prior to washing, lysis, and nonreducing SDS-PAGE, as described previously. Upon probing the membranes with W6/32, a slower migrating product appeared with increasing concentration of BMC (Fig. 4A,*). Although this species ran at a similar position to the HLA-B2705 conformer in Figure 2A, this population of MHC class I molecules was boiling resistant (compare lanes 7 and 8). The expression levels of PDI itself were unchanged by treatment with BMC (Fig. 4B, lanes 1–4). We conclude from this experiment that at least some W6/32 reactive HLA-B populations are redox dependent (but sequence independent), and that the conformation of MHC class I molecules can be manipulated using a PDI mimic.

DISCUSSION

Our data show that the oxidation of MHC class I molecules is complex and dependent on context as well as sequence. When HLA-B2705 heavy chains are transfected into adherent cells, high expression levels result in oxidative misfolding, and this does not depend on any individual free cysteine residue (Fig. 1). We found that BiP associated with wild-type HLA-B2705 heavy chains as well as the cysteine mutants C67A, C308A, and C325A. Previous reports have shown that HLA-B27 wild type and C67S heavy chains associate particularly strongly with BiP in cell lines and in animal models of AS (4, 27). We now show that this is also the case for two other HLA-B27 cysteine mutants. Although the physiological significance of BiP association with HLA-B27 is unclear, it may predispose cells to an unfolded protein response (UPR) by titrating BiP away from UPR activators such as Ire1 (15).

Mis-oxidation due to high expression is likely to be the cause of arthritic disease in some rat and mouse models of AS (25). In these animals, different levels of expression can lead to different disease outcomes, and the expression of compatible β2m can reduce disease severity (14, 24). Thus having sufficient β2m expression to avert extensive intermolecular HC disulfides is important, but is not the only factor involved in HLA-B27 oxidative folding and quality control. Endogenous HLA-B27 molecules can oxidize differently, as we have shown for HLA-B2704 and HLA-B2705 (Fig. 2). Although Tran et al. tested the reactivity of W6/32 to a number of nonreduced, partly denatured B alleles, the behavior of HLA-B2704 has not been reported (26). Our results show that minor sequence differences between the two alleles can have a major impact on an MHC class I molecules conformation, and that redox-dependent structures can depend (probably indirectly) on other amino acids than cysteine (Fig. 2). These results cannot be explained by an absolute loss of W6/32 reactivity, since we can retrieve different W6/32 reactive structures with BMC (Fig. 4) and W6/32 is known to immunoprecipitate all peptide loaded HLA-B complexes. HLA-B2704 and HLA-B2705 differ by just three residues (Fig. 5). Perhaps the amino acid differences around the C67 residue and the disulfide bond close to the peptide-binding groove influence the oxidation pathway of HLA-B27 and/or its association with β2m. The conformational difference between HLA-B2704 and HLA-B2705 must also rely, at least indirectly, on a disulfide bond, because of its visualization in nonreducing gels. Whether this is due to a heavy chain disulfide or β2m involvement in an SDS-resistant complex requires further investigation. However, the nature of differing epitope recognition by antibodies makes these investigations complicated. We have not been able to detect β2m-HC complexes in nonreducing gels using two different β2m antibodies, and although β2m immunoprecipitates contain material that reacts with W6/32 after nonreducing SDS-PAGE, we cannot exclude that β2m has been lost from the complex during electrophoresis (Fig. 3). However, it is clear that the HLA-B2704 molecules that react with W6/32 after nonreducing SDS-PAGE are capable of assembling with β2m and presumably peptides.

It has been suggested that residue 116 of HLA-B27 is important in determining the susceptibility to AS, given that HLA-B2705 (linked to AS) and HLA-B2709 (not linked to AS) differ solely at this position. Indeed, the conformations of these two allelic products in complex with the same EBV LMP2-derived peptide (RRRWRRLTV) are different, suggesting that molecular mimicry can be allele (context) dependent (10). In particular, the presence of H116 results in the formation of different heavy-chain/peptide salt bridges, and leads to increased flexibility in the peptide binding groove compared to HLA-B2705 (13). Our results show that there is also a position 116 independent conformational change between HLA-B2704 and HLA-B2705 that must involve one or more of the HC residues 77, 152, and 211. The role of S77 in HLA-B2704 is worth investigating more closely, given the

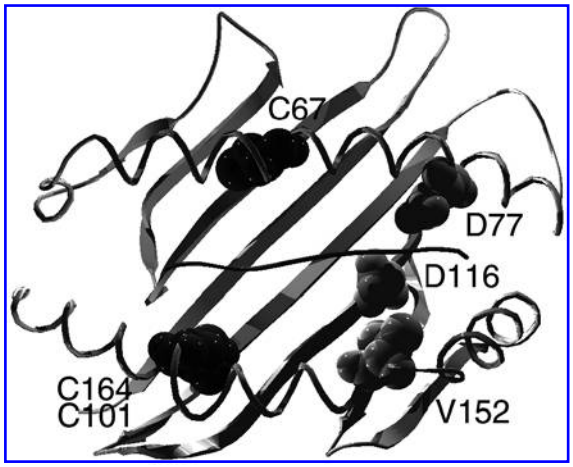

FIG. 5. Structure of the HLA-B2705 peptide binding groove. The peptide binding groove of HLA-B2705 (coordinates from PDB deposit 1JGE) was depicted using Swiss Pdb viewer 3.7 (http://us.expasy.org/spdbv) and rendered in POV-Ray (www.povray.org). The free cysteine C67 and the disulfide bonded amino acids C164 and C101 are shown as space filling residues, along with D116 (H in HLA-B2709), D77 (S in HLA-B2704), and V152 (E in HLA-B2704).

potential of D77 to form a salt bridge with antigenic peptides in HLA-B2709 (when H116 is present).

We suggest that the relative susceptibility of HLA-B27 HC to conformational change is likely to depend on a number of factors, not just the unpaired cysteine residue C67. The charge of the amino acids in the surrounding sequence, the peptides bound in the groove, and the extrinsic nature of the local redox environment could all contribute. The involvement of multiple factors could explain why some C67 containing alleles are not linked to AS, and why HC position 116 can influence disease susceptibility. In this respect, it will be interesting to examine lymphocytes from HLA-B2705 and HLA-B2704 positive individuals with and without AS, to determine the nature of the W6/32-reactive and oxidised HC forms.

We have shown here that we can manipulate the conformational state of HLA-B27 (the W6/32-reactive pool) by using the redox reagent BMC (Fig. 4). *In vivo*, the HC redox environment could be controlled either by small thiol-containing compounds or by the expression of protein catalysts such as PDI, ERp57, or ER oxidoreductases, which could change upon UPR induction or during bacterial infection. Further experiments will now be required to determine the effects of the redox environment upon AS, and to design more selective and MHC class I-specific reagents to manipulate the HC oxidation state.

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ABBREVIATIONS

AS, ankylosing spondylitis; β_2m, beta 2 microglobulin; BMC, 1,2-bis(2-mercaptoacetamido)cyclohexane; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EBV, Epstein-Barr virus; ECACC, European collection of cell cultures; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; FCS, fetal calf serum; HC, heavy chain; HLA, human leukocyte antigen; MHC, major histocompatibility complex; NEM, *N*-ethylmaleimide; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; SDS, sodium dodecyl sulfate; TAP, transporter associated with antigen processing.

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