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Refolding of HLA-B27 heavy chains in the absence of β_2m yields stable high molecular weight (HMW) protein forms displaying native-like as well as non-native-like conformational features: Implications for autoimmune disease

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Abstract

Refolding of the heavy chain of the Class I HLA molecule, HLA-B27, in the absence of β_2m, yields soluble high molecular weight (HMW) oligomers reminiscent of the oligomeric forms of β_2m-free heavy chains (FHCs) of class I HLA antigens observed on cell surfaces. Here we examine the structural characteristics of HMW B27 in respect of features potentially relevant to autoimmunity, such as: (a) retention of native-like structure, since this could facilitate non-canonical interactions with T-cell receptors even in the absence of bound β_2m and peptide, or (b) presence of non-native structure, since this could yield novel (non-self) antigenic conformational epitopes that could elicit immune attack. We report that HMW B27 is characterized by high secondary structural content, structural stability, stability to proteolysis by trypsin, and structural features that are both partly native-like, and partly non-native-like, as assessed through the binding of conformationally-distinguishing and cross-reacting scFv antibodies specifically selected against HMW B27. We also present cell ELISA data with conformation-specific scFv antibodies that distinguish between lymphocytes from individuals who are healthy and B27 positive, and those who are B27 positive but suffering from ankylosing spondylitis.

Keywords: HLA-B27; MHC dysfunction; Protein misfolding; Cross-display; Secondary structure; Thermal stability; Autoimmunity

1. Introduction

MHC Class I heavy chains have classically been known to be unfolded and degraded in the absence of bound β_2m [1]. During the last decade, however, data have emerged to suggest that β_2m-free heavy chains (FHC) exist on the surfaces of all MHC class I-expressing cells and, in particular, on the surfaces of activated lymphocytes, transformed lymphoblasts, fibroblasts and neuroblastoma cell lines [2–7]. Cell-surface FHCs have been reported to show relatively long half-lives of several hours [4]. Of course, the functional role played by such FHCs, if any, remains to be discovered. Even so, what is known already is that FHCs tend to cluster, or oligomerize, on cell surfaces [6,8]. It has been suggested that these clusters may be HLA sinks that act as sources of soluble HLA, through cleavage by metalloproteinases [9]. It has also been suggested that FHCs may perform some sort of an immunomodulatory role [10].

Of the various types of class I HLA molecules, one that has been specifically observed and examined both *in vitro* and *in vivo* in the absence of β_2m is HLA-B27. Disulfide-bonded homodimers of β_2m-free HLA-B27 have been shown to form *in vitro* [11], and have also been detected on the cell surface and in the endoplasmic reticulum [12–14]. Structurally-distorted peptide-binding clefts retained within such β_2m-free B27 homodimers have been suggested to bind and display peptides to γ and δ chain TCRs [15]. Separately, a particular subtype of

Abbreviations: MHC, major histocompatibility complex; HLA, human leucocyte antigen; HMW, high molecular weight; TC, ternary complex; scFv, single chain variable fragment

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B27, known as HLA-B2705, has also been discovered on the cell surface in a β_2m-free but peptide-bound state [16].

In addition to homodimers, B27 chains have also been observed to form soluble high molecular weight (HMW) forms *in vitro*. Indeed, the bulk of any B27 heavy chain population undergoing folding in the absence of β_2m tends to adopt HMW forms, with disulfide-bonded homodimers constituting only a minority population [11]. Still, homodimeric B27 has attracted the larger share of research attention, presumably because the HMW forms have commonly been taken to be simply aggregates that are intractable to conventional structural-biochemical analyses. It appears, therefore, that whereas a whole new paradigm could be taking shape in relation to our understanding of the structure- and function-related behavior of β_2m-free HLA chains in general, and β_2m-free HLA-B27, in particular, there remains a lacuna in our knowledge concerning HMW forms despite occasional comments about the possibility of this form being structured [11].

Assuming that HMW oligomers observed *in vitro* could be analogs of FHC oligomeric clusters seen *in vivo*, and given that FHCs are ubiquitous and clearly somehow functionally important, we considered it important to create and examine HMW B27 through biophysical and biochemical investigations, to focus attention on HMW B27, a molecule typically associated with autoimmune disease, and see: (i) whether it is structured and conformationally stable, and (ii) examine it in relation to its native-like and non-native-like structural content, with a view to exploring potential diagnostic indicators of misfolding associated with disease.

2. Materials and methods

2.1. Genetic manipulations

A vector expressing HLA-B2705 (plasmid pLM1-HLA-B27, encoding the extracellular domain of HLA-B27) was obtained as a gift from Dr. D. N. Garboczi (NIH, USA). We modified the vector genetically to engineer the inclusion of an N-terminal 6xHis tag in genetic fusion with the gene encoding HLA-B27, to create a modified vector, pHisB27, as follows: The gene encoding extracellular B2705 was amplified using the primers 5′-TATATTAATTAGAATTCAGGAGGAATTTAAAATGCATCACCATCACCATCACGGCTCT-3′ and 5′-GTAGGCGTCCTGGTGGTACCCGCGGAGG-3′ in a PCR reaction carried out under the following conditions: denaturation for 3 min at 94 °C, followed by 25 cycles of 1 min denaturation at 94 °C, 1 min annealing at 46 °C, 2 min extension at 72 °C, and then by a further 15 min of extension at 72 °C. This reaction amplified a (380 bp) section of the gene, beginning at its 5′ end, terminating at a KpnI site located 380 bp downstream of its 3′ end (in the plasmid pLM1-HLA-B27), and introducing new codons encoding 6 contiguous histidine residues immediately 5′ of the original codon encoding the first residue. The product thus obtained was digested with EcoR1 and Kpn1 and cloned between the same two sites on a double-digested plasmid pLM1-HLA-B27. This plasmid, renamed pHisB2705, was then transformed into *E. coli* BL21(DE3)pLys, and clones containing inserts (and producing protein upon induction with IPTG) were identified through SDS-PAGE analysis. A protein-producing clone was subjected to DNA sequencing on an Applied Biosystems 310 Prism automated sequencer to verify the sequence and the correct incorporation of the 6xHis residues at the N terminus of the protein.

Similarly, the DNA encoding human β_2-microglobulin was subcloned from the vector (pc DNA 3.1) obtained as a gift from Dr. F. Greten, Germany, and modified to incorporate a 6xHis tag. Using primers, 5′-GCTACCGCATGCATCCAGCGTACTCCAAAGATTCAGG-3′ and 5′-GCTAGGAAGCTTTTACATGTCTCG-3′, PCR was performed with the following program: 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, with an additional extension of 5 min at 72 °C. The PCR product of 356 bp was digested with Sph1 and HindIII and ligated into the vector pQE-30, pre-digested with Sph1 and HindIII and transformed into competent XL1-Blue *E. coli*; DNA sequencing was done to confirm the integrity of the construct and further transformation of this DNA was carried out into competent M15-pREP4 *E. coli* cells for overexpression.

2.2. Protein expression and purification

A 500 ml culture set up with an innoculum of cells transformed with pHisB2705 was incubated at 37 °C, and induced to produce protein by addition of IPTG (0.5 mM) at $O.D_{600}$ of 0.4. Cells were harvested by centrifugation, and the cell pellet was resuspended in 0.1 M NaH_2PO_4, 0.01 M Tris–Cl, pH 8.0 buffer containing 8 M urea. After stirring for 1 h at room temperature, the suspension was centrifuged and the supernatant was loaded onto a Ni-NTA column pre-equilibrated with the same buffer. Elution of bound protein was done with 0.1 M NaH_2PO_4, 0.01 M Tris–Cl, pH 4.5 buffer containing 8 M urea. Refolding of the protein was carried out by dialysis against progressively lower concentrations of urea in 0.1 M NaH_2PO_4, 0.01 M Tris–Cl buffer, pH 8. This purified protein was subjected to N-terminal sequencing on an ABI 377 protein sequencer, to confirm chemical identity. β_2m was expressed and purified in a similar manner.

2.3. Nondenaturing (native) polyacrylamide gel electrophoresis

HMW B27 samples were prepared for non-denaturing polyacrylamide gel electrophoresis through treatment with various combinations of heat denaturation (boiling or not boiling samples), SDS denaturation (addition, or omission, of 1% SDS in the sample loading buffer), and reduction (addition, or omission, of 5 mM DTT). There was no SDS included in the gel casting buffers or in the running buffer used for electrophoresis.

2.4. Formation of complexes of HLA-B27 with β_2m and peptide

Incubation of HLA-B27 heavy chain (HC), β_2-microglobulin and peptide in the molar ratio of 1:2:10 (31 μg/ml:24 μg/ml:10 μg/ml) was carried out in 100 mM Tris–Cl, 400 mM Arg, 2 mM EDTA, 0.5 mM PMSF pH 8.0 at 10 °C, for 36–48 h, as described by earlier authors [17]. The peptide incorporated into the complex was a nonameric peptide, N-KRWHMGLNK-C. As per the epitope prediction program developed by Rammensee *et al.* (http://www.syfpeithi.de/) [18], this peptide sequence is predicted to be capable of forming a high-affinity complex with HLA-B27 and β_2m, with a score of 26. This score falls at the boundary of values [26–27] characterizing high-affinity binding peptides. Analysis of complex formation, and purification of complexes was carried out by gel filtration chromatography, using a Superdex 200 gel filtration column. Separately, we subsequently also prepared complexes of B27 with two other high-affinity binding peptides, N-KRWIIMGLNK-C and N-RRYLENGKETL-C.

2.5. Chromatography

The purified refolded B27 protein in soluble form, as well as control complexes of B27 with β_2-microglobulin and peptide, were analyzed through gel filtration chromatography on a Superdex 200 column attached to a Pharmacia SMART System, using 150 mM NaCl and 20 mM Tris-Cl buffer, pH 8.0 for equilibration. The gel filtration experiment was repeated in the presence of DTT (5 mM) to examine the presence of disulfide-bonded species.

2.6. Spectroscopy

UV-visible absorption spectra were collected on a Varian 50-Bio spectrometer. In the case of the HLA-B27, an absorbance of 2.07 at 280 nm (OD_{280}) was taken to indicate a concentration of 1 mg/ml as per the predicted extinction coefficient. Similarly, in the case of β_2m, an absorbance of 1.49 was taken to indicate this concentration. A Perkin Elmer LS-50B spectrofluorimeter was used to examine fluorescence characteristics, with excitation and emission band passes of 5 nm each, with excitation being carried out at 280 nm, and with monitoring of emission in the range of 300 to 400 nm. A Jasco J710 spectropolarimeter was used to collect CD spectra, at controlled temperatures, through a peltier-based heating/cooling arrangement. In case of far-UV CD measurements for HMW B27, scanning was done between 250 and 190 nm,

using a protein concentration of 0.1 mg/ml and a cuvette of 0.2 cm path length. The complex of HLA-B27 with β_2m and peptide was scanned in the region of 250–200 nm, using a cuvette with path length of 0.1 cm. Thermal melting was carried out by heating the proteins at a rate of either 2 or 3 °C/min, and monitoring changes in the CD signal at 222 nm, with cooling also performed using the same temperature gradient. Measurement of the CD spectrum at 95 °C or 99 °C was done after the samples were equilibrated at this high temperature for 5 min (adequate for chemical, thermal and conformational equilibration of the sample).

2.7. Digestion of protein with trypsin

HLA-B27 was incubated with trypsin at 37 °C, using a trypsin: B27 molar ratio of approximately 1:6000 for a period of 1, 2 and 3 h and one sample was incubated overnight. The polypeptide fragment, found to be relatively resistant to proteolysis was subjected to N-terminal sequencing on an ABI 377 sequencer.

2.8. ELISA of HMW B27

100 μg/ml each of HMW B27 as well as the complex were coated on an ELISA plate; separately control proteins (BSA and ovalbumin) were also coated onto the ELISA wells using solutions of the same concentrations. Mouse monoclonal w6/32 antibody (Abcam cat no. 23755) was used at a concentration of 1:30 (stock concentration being 1 mg/ml). Anti-mouse HRP-labeled secondary antibody (Sigma; 1:500 dilution) was used; TMB was used as the substrate.

2.9. Screening of scFv phages from phage library

Two phage-display human antibody scFv libraries (Tomlinson I and J, procured from MRC, UK), constructed in fusion with the pIII protein of M13 filamentous phages, were used to screen for antibodies showing affinity to HMW B27. First, immunotubes (Nunc) were coated with 4 ml of HMW B27 of 100 μg/ml concentration; the unbound protein was washed using PBS, followed by blocking with 10% MPBS for 2 h. After 3 washes with PBS, 10^{12} phages of the scFv I or J library were added to immunotubes and incubated for 60 min at room temperature. PBS containing 0.1% Tween-20 was used for washing. Bound phage was eluted with 500 μl of trypsin-PBS. *E. coli* TG1 was infected with the eluted phages and an aliquot was serially diluted and plated on 2× TY agar containing 1% glucose and 100 μg/ml ampicillin for titration of phages. The remaining TG1 culture was centrifuged and the pelleted bacteria resuspended in 50 μl of 2× TY and plated on a TYE plate containing 1% glucose and 100 μg/ml ampicillin. Colonies were collected and resuspended in 2× TY/15% glycerol and used to prepare phage for the next round of selection. Three rounds of such selection through 'bio-panning' were performed on the HMW form of the HLA-B27 heavy chain. The recovery rate of the third round bio-panning was close to that of the second round indicating that positive clones were fully enriched and as expected, an enrichment within the range of 10–100 fold was observed after subsequent bio-panning. Monoclonal phage ELISA was performed with 96 clones as follows, to confirm whether phages obtained indeed displayed B27-binding scFvs: After *E. coli* TG1 was infected with scFv phages derived from the third round of bio-panning, and plated on 2× TY agar, individual colonies were inoculated into a 96-well round-bottomed microtiter plate, grown overnight, and phage prepared as previously described (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf). Then, a 96-well flat-bottomed microtiter plate was coated with 100 μg/ml HLA-B27 in PBS overnight at 4 °C. Excess protein was washed away thrice and the plate blocked with milk-containing PBS for 2 h at 37 °C. A total of 150 μl of each phage-containing supernatant was transferred to the HMW B27 coated plates and incubated with shaking for 2 h at room temperature. The plate was washed four times with PBS/0.1% (v/v) Tween-20 and four times with PBS. Then, 100 μl anti-M13/HRP monoclonal antibody (Amersham, UK) was added with shaking to each well at 1:5000 dilution in 10% MPBS and incubated shaking at room temperature for 1 h. The wells were washed with PBS/0.1% Tween-20 and PBS before the use of a TMB substrate-peroxidase kit to visualize phage binding through color development. Reactions were stopped by addition of 1 M sulfuric acid and absorbance was measured at 450 nm with a background correction of 650 nm. Fig. S1 (Supplementary data) shows that most of the 96 scFv-displaying phage clones selected from libraries I and J showed considerable binding to HMW B27.

2.10. Monoclonal scFv ELISA

The selected phages from the 96 clones were used to infect HB2151 (a non-suppressor strain). The phage genomes in this cell function as phagemids. Induction with IPTG under standard conditions leads to expression of soluble scFv antibody fragments, through the action of an amber-suppressor stop codon placed between the scFv-encoding region and the phage pIII coat protein region, within the fusion construct encoded by each phage genome. The expressed

Table 1
Details of the binding capabilities of the 20 selected clones from libraries I and J respectively

1	2	3	4	5	6	7	8	9	10
scFv ID	Raised against	HMW ELISA (H)	Ternary complex ELISA (TC)	HMW/complex (H/TC)	Western blot+ve (W)	Sequence epitope H and TC	Sequence epitope H only	Conf. epitope H and TC	Conf. epitope H only
I1H	HMW	2.74	1.61	1.7:1	√	√			
I2A	HMW	2.78	0.35	8.0:1	√	√√			
I2C	HMW	2.87	0.34	8.4:1	√		√√		
I2H	HMW	2.69	0.25	10.7:1	√		√√		
I3A	HMW	2.79	1.00	2.8:1	√	√			
I3G	HMW	2.68	0.20	13.4:1					√√√
I3H	HMW	2.35	0.09	26.1:1	√		√√√		
I4B	HMW	2.61	0.33	7.8:1				√√	
I5A	HMW	2.72	1.05	2.6:1				√	
I9D	HMW	2.36	0.278	8.8:1					√√
I11E	HMW	2.76	1.41	2.0:1	√	√			
J1A	HMW	2.31	0.32	7.2:1				√√	
J2A	HMW	2.51	1.36	1.8:1	√	√			
J2B	HMW	2.85	0.76	3.8:1	√	√			
J3E	HMW	2.47	0.06	39.1:1	√		√√√√		
J3F	HMW	2.61	1.00	2.6:1	√	√			
J3H	HMW	2.47	2.09	1.2:1	√	√			
J5E	HMW	2.76	0.98	2.8:1	√	√			
J6F	HMW	2.67	0.69	3.9:1	√	√			
J8D	HMW	2.06	0.19	11.0:1					√√

scFvs were obtained through preparation of the periplasmic extracts from each phagemid-containing population, and extract supernatants were used to perform ELISA (with the HMW form of HLA-B27 coated on the wells of the ELISA plate, as described above) and the detection of bound scFv was performed using Protein-A HRP conjugate.

2.11. Cell-ELISA

Peripheral blood mononuclear cells (PBMCs: 100 μl of a suspension of 2×10^6cells/ml) were added to poly-L-lysine-coated wells in a 96-well plate. After three washings with 1× PBS, cells were fixed using absolute methanol for 5 min at room temperature. Internal peroxides were inactivated by treatment with 0.3% hydrogen peroxide in absolute methanol for 30 min. This was followed by 3 washings with 1× PBS; wells containing cells were blocked by using 5% BSA in 1× PBS overnight at 4 °C. Primary antibody (scFv) was added into wells after washing with 1× PBS and incubated for 2 h at room temperature. Mouse anti-His monoclonal antibody at a dilution of 1:1000 was added to each well and incubated for 2 h. This was followed by incubation with anti-mouse HRP-conjugated antibody (1:1000 dilution) for 1 h at room temperature. Color was developed with TMB; the reaction was stopped with 1 N sulfuric acid, and the plates were read at 450 nm.

2.12. Patients

Standard serological and DNA-based testing is utilized in our laboratory as part of a routine clinical service offered to patients for identification of HLA Class I and Class II subtypes. Such testing allowed us to identify B27 positive patients who had earlier been clinically diagnosed to have ankylosing spondylitis. PBMCs were collected from five B27 patient volunteers suffering from the disease, for cell ELISA testing with each scFv antibody from a panel of about 12 antibodies. The results reported for each antibody thus show the mean and error bars for five separate samples from five distinct B27 positive individuals diagnosed to have ankylosing spondylitis. Separately, B27 negative individuals were identified from amongst healthy volunteers being tested for their HLA Class I subtype. PBMCs were collected from five healthy B27 negative individuals for cell ELISA testing, as negative controls, and the results reported are for five distinct samples. However, in regard to the samples for B27 positive individuals who are diagnosed to have not yet developed the disease, it may be appreciated that such individuals are difficult to come by in a hospital environment. Therefore, the five samples were drawn from only three healthy individuals who were clearly asymptomatic but found to carry the B27 antigen, as healthy B27 positive controls for cell ELISA testing, with PBMCs from two of these individuals being used on more than one occasion. The procedures involving patients (including issues concerning informed consent) were followed after ratification by the institute ethics committee.

2.13. Statistics

Five independent experiments were performed for cell ELISA studies with each of the four antibodies, I3G, I9D, J2A and I3H. Data were expressed as mean±standard deviation. Student's unpaired *t*-test was performed for comparison of data points, with p less than 0.05 being considered to be significant.

3. Results

When recombinant β_2m-free B27 samples are prepared under conditions favoring oxidative formation of disulfides, homodimeric B27 constitutes only a minor fraction (<10%) of any such β_2m-free B27 population, with most of the folded population consisting of high molecular weight (HMW) species [11]. This HMW material has not been previously tested to examine whether it contains structured chains resembling those within either FHC clusters, or within regular heterotrimeric β_2m- and peptide-containing B27 complexes on cell surfaces.

3.1. Non-disulfide dependent existence of HMW species

As seen in Fig. 1A, gel filtration analysis carried out after refolding of chains purified under denaturing conditions shows that no homodimeric population is detectably formed in the refolded protein through simple air oxidation. Instead, the entire protein population elutes at ~0.95 ml from a SMART Superdex

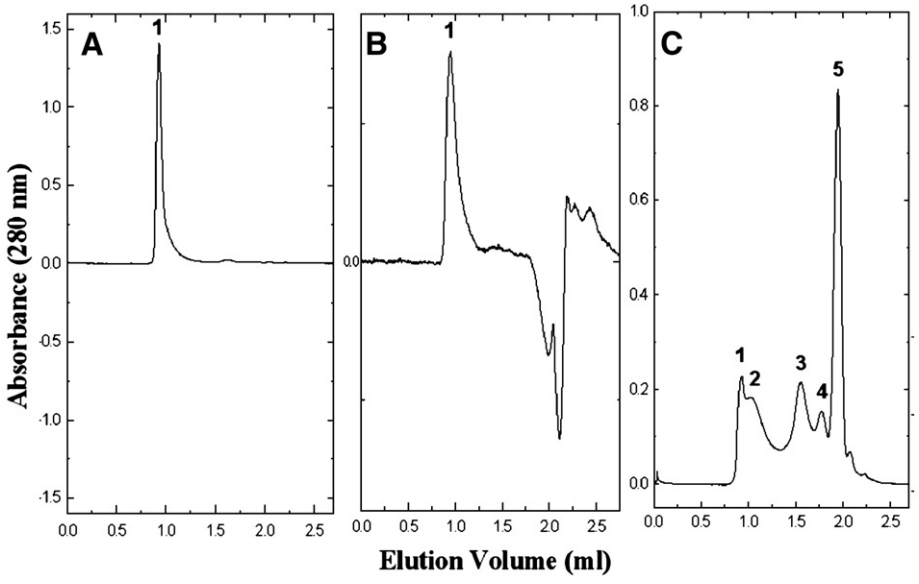

Fig. 1. Gel filtration chromatograms of HMW B27 obtained on an analytical Superdex 200 column in the absence (panel A), and presence (panel B) of 5 mM DTT, demonstrating that the HMW population does not unfold/dissociate upon reduction of disulfide bonds with DTT. The dips in the chromatogram at ~2.0 ml elution volume in panel B owe to minor differences in the DTT concentration in the sample and running buffer. The gel filtration chromatogram of the complex of HLA-B27 with β_2m and peptide on the same column (panel C) is characterized by elution of the complex at 1.54 ml (peak 3) corresponding to a molecular weight of 47 kDa. HMW B27 is designated by peak 1, HMW β_2m by peak 2, while peaks 4 and 5 represent monomeric and low association-status β_2m.

200 column. Since the void volume of this column is ~0.85 ml, this suggests a molecular weight ≤300,000 Da for HMW B27. It is with this HMW protein population that further work reported in this paper was carried out.

HMW B27 was equilibrated with 5 mM DTT and chromatographed on the same Superdex 200 column pre-equilibrated with buffer containing 5 mM DTT. As Fig. 1B shows, the presence of this high concentration of DTT did not result in elution of smaller molecular weight species; rather, elution continued to be seen close to the void volume of the column, at the exact same elution volume earlier obtained without DTT. The dip seen in the later section of this chromatogram (at 2.0–2.1 ml) can be ascribed to the negative absorption resulting from there being a marginally higher concentration of DTT in the running buffer as compared to the sample, despite efforts to make both equivalent to 5 mM DTT. Generally, DTT concentrations of 1–2 mM are sufficient to reduce all the disulfides in a protein, and a concentration of 5 mM DTT is used for the reduction of partly buried disulfides (although, of course, fully buried disulfides could resist reduction even with this concentration of DTT). The fact that the HMW species does not disassemble in the presence of 5 mM DTT to release any distinct and detectable lower molecular weight forms (eluting at volumes ≥0.95 ml) suggests that even if disulfide bonds exist within HMW B27 when it is formed, such bonds may not be critical for the adoption and retention of the oligomeric form. To test this further, we carried out another set of experiments in which HMW B27 was subjected to electrophoresis under both denaturing (SDS-PAGE) and non-denaturing (native) conditions after being prepared for electrophoresis through treatment with different combinations of inclusion, or omission, of a denaturing agent (1% SDS), a reducing agent (5 mM DTT) and heat (boiling, or lack thereof). The results are shown in Fig. S3 (Supplementary data). Briefly what electrophoresis assays is whether the HMW B27 remains in the well of the gel, or at the interface of the stacking and resolving gels unable to enter the gel when no denaturing, or reducing, influences are used (i.e., whether it is a soluble aggregate), and also whether it enters the gel to migrate at its expected position, when denaturing and/or reducing influences are used. In both native and SDS-PAGE experiments, inclusion or omission of 1% SDS in the sample buffer was used, regardless of the fact that the running buffer in SDS-PAGE contains a lower concentration of SDS (0.1%) whereas no SDS is present in native electrophoresis. The results show: (i) SDS alone cannot disassemble the HMW form of B27 (lanes A2, A3, B2 and B3); (ii) when SDS is used in combination with DTT, HMW is dissociated to a significant extent even without boiling of samples (lanes A6 and B6); (iii) DTT treatment needs to be combined with either SDS treatment (lanes A6, B6) or with boiling of samples (lanes A9, B9) to effect a significant extent of dissociation of HMW B27. Based on the chromatographic and electrophoretic data, therefore, it may be concluded that noncovalent interactions such as hydrophobic interactions, electrostatic interactions and hydrogen bonding must also play a role in holding the HMW form together, in combination with disulfide bonding. To summarize this result, we do not propose that our gel filtration and electrophoretic data excludes the existence of disulfide bonds within HMW B27; the data merely establishes that reduction of disulfides does not lead to complete disassembly of HMW B27 into smaller forms unless reduction is combined with some other structurally-destabilizing influence such as heat or a denaturant like SDS. Thus, we conclude that HMW B27 must be predominantly stabilized by noncovalent interactions. As to the nature of such interactions, we have earlier speculated that HMW B27 could consist of structured B27 chains (possessing native-like structure), which bind, in intermolecular fashion, to the residues 169–181 of neighboring B27 molecules [19]. These and other models will doubtlessly be tested, as HMW B27 is studied further. As a control, it may be noted that in contrast to the behavior of HMW B27, the regular trimeric complex of B27 with β_2-microglobulin and a nonameric peptide (see Materials and methods) elutes at 1.54 ml (Fig. 1C) corresponding to a molecular weight of 47 kDa.

3.2. Inordinately high secondary structural content in HMW B27

The far-UV CD spectrum of the β_2m-free HMW B27 is shown in Fig. 2A. The protein is clearly seen to have a spectrum characteristic of very well formed secondary structure, with

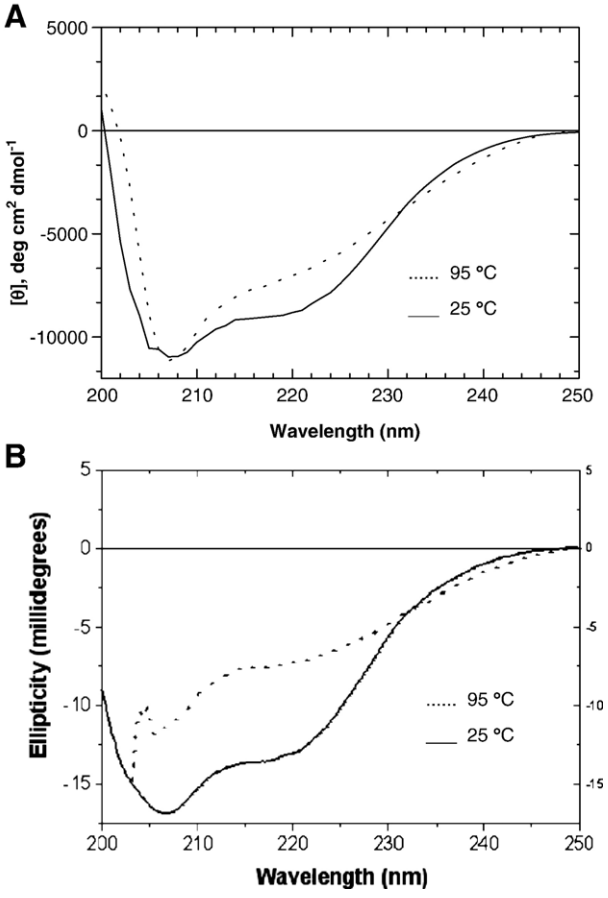

Fig. 2. Far-UV CD spectra of (A) HMW-B27 at 25 °C (—), and 95 °C (- - -), demonstrating that the protein has an inordinately high secondary structural content, as well as high thermal stability. (B) trimeric complex of HLA-B27 with β_2m and peptide at 25 °C (—), and 95 °C (- - -), clearly showing that the complex undergoes thermal melting in response to increase in temperature.

evidence of formation of both α helices (detected through the presence of negative band maxima at 222 and 208 nm) and β sheets (detected through measurement of the intensity of the negative signal at 218 nm, relative to the intensity at 222, and 208 nm). For comparison, the far-UV CD spectrum of the B27 heavy chain, in complex with bound peptide ligand and β_2m is shown in Fig. 2B; it may be noted however that as the estimation of protein concentration in solutions of the complex cannot be done accurately, the data for the complex is shown in terms of raw ellipticity, rather than as mean residue ellipticity. The crystallographically-determined structure of the trimeric HLA B27 complex is known to contain both types of secondary structure [20]. Structural analysis reveals that about 82 out of 276 chain residues are α helical (i.e., about 30% of all residues), whereas about 126 residues make up strands that go to make β sheets (i.e., about 45% of all residues). The remaining residues form β turns, and various other structures including both ordered (but irregular) and disordered structures or 'randomly coiled' structures. To compare these figures to the estimated secondary structural content of HMW B27, we analyzed its CD spectrum after conversion of data to mean residue ellipticity. It may be noted here that rigorous analysis of such a CD spectrum, done in order to determine the percentages of chain length constituted by various secondary structures, ordinarily requires the availability of ellipticity data in the full range of 250–180 nm. However, data over this entire range is difficult to obtain for such samples, because HMW species scatter short wavelength radiation very effectively on account of their multimeric status and large hydrodynamic volume, leading to unacceptably poor signal/noise ratios in the 190–180 nm region. Fortunately, however, methods which work with data collected over smaller ranges are available; therefore, we attempted analysis using data collected between 250 nm and 190 nm, using a neural network-based approach available as a service on the internet [21]. The CD spectrum at 25 °C suggests that, to a first approximation, HMW B27 has over 50% of its residues in α helical structure, with the remaining chain length adopting β sheets and other structures. This indicates that the B27 chain has somewhat more helical structure in the HMW species formed in the absence of β_2m (50% of the chain) than in the presence of β_2m (30% of the chain, as stated earlier), which definitely rules out any net lower folded status resulting from the absence of β_2m.

Where does this extra helicity come from? We suspect that tracts of one of the β sheet-rich regions of B27 that are no longer engaged in interactions with β_2m may have undergone transformation into helical structure(s), possibly aided by the proximity of other misfolded B27 chains in the HMW species. Since, at least in principle, it remains possible for secondary structural content to exist in a chain even without the existence of widespread tertiary structural contacts (especially as seen in molten globule-like conformations), it was necessary to further examine these HMW species for tertiary structural content.

3.3. HMW B27 has tertiary structure

The fluorescence emission spectrum of β_2m-free B27 is shown in Fig. 3. As is well-known, the wavelength of maximal emission (λ_{max}) of a protein is a measure of its folded or unfolded state. The indole side chain of the dominating fluorescent residue, tryptophan (Trp), is extremely sensitive to the polarity of its immediate environment. Thus, Trp emission displays a blue shifting of λ_{max} from the characteristic 353 nm shown by free Trp (or by Trp exposed to aqueous solvent, e.g., in unfolded proteins), to significantly shorter wavelengths upon burial of Trp residues within the interior of a folded protein. A blue-shifted λ_{max} is diagnostic of burial of tryptophans accompanying structure formation. Fig. 3 shows that HMW B27 has a λ_{max} of 346 nm whereas molten-globular HMW B27 may have been expected to have a λ_{max} of 353 nm, just like any unfolded protein. Therefore, emission of HMW B27 at 346 nm is clearly indicative of the existence of tertiary structure. The question that now arises is whether this tertiary structure of HMW B27 is native-like, to any degree.

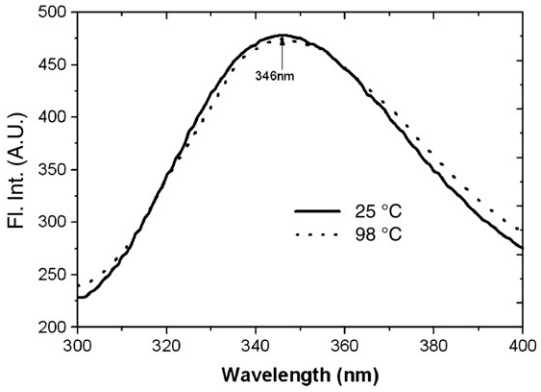

Fig. 3. Fluorescence emission spectrum of the HMW HLA-B27 at 25 °C (—), demonstrating that burial of tryptophan residues in the protein's interior leads to its emission peaking at 346 nm, rather than at 353 nm as might have been expected for a form lacking tertiary structure. The same spectrum at 98 °C (- - -) shows no change in emission wavelength, confirming the retention of tertiary structure even at high temperatures. The *y*-axis shows the fluorescence emission intensity in arbitrary units as is standard practice in fluorescence spectroscopy.

3.4. The tertiary structure of HMW B27 is partly native-like and partly non-native-like

Short of determining the actual three-dimensional structure of a protein by X-ray crystallography, the best approach to examine it for native-like structural content is to probe it with conformation-specific antibodies that bind only to native-like conformations (preferably adopted by discontinuous stretches of polypeptide sequence) and not to sequence-specific epitopes that can be bound even within a grossly unfolded antigen. One such antibody, described well in the literature, is w6/32. This antibody binds to a structural epitope constituted of sequences assembled from the alpha 1 and/or alpha 2 domains of the Class I MHC chain [13,22], and is thought to bind only to Class I MHC polypeptides in native form as a part of a complex with β_2m [23]. As the histogram in Fig. 4 shows, we find that w6/32 binds to HMW B27, indicating that the w6/32 antibody-binding regions of the α1 and α2 domains are folded as well as correctly assembled. As is well-known, these two domains together give rise to the peptide-binding cleft in MHC. It may be noted that

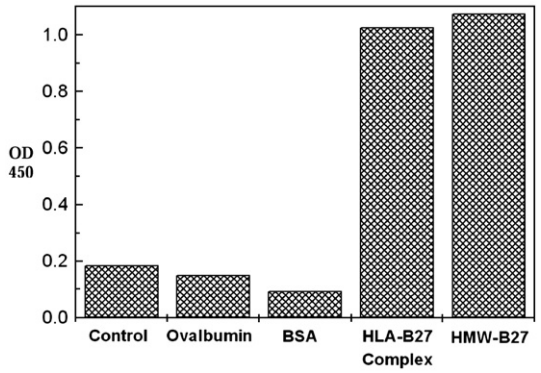

Fig. 4. The histogram represents results of ELISA, using the antibody w6/32 against a control sample (no addition of antibody), two non-specific proteins (ovalbumin and BSA), the HLA-B27 complex, and HMW-B27. The figure shows very efficient binding of this well-known conformation-specific antibody to HMW B27 as well as to the trimeric complex of HLA-B27 with β_2m and peptide, indicating that the alpha 1 and alpha 2 domains are folded as well as correctly assembled in a native-like conformation. The *y*-axis shows the optical density at 450 nm.

w6/32 has been also seen to bind to the disulfide-bonded, homodimeric, β_2m-free form of B27, with this binding being taken to be evidence that the homodimer maintains some conformation of its peptide-binding groove [11,24]. Clearly, therefore, by the same criterion, the binding of the w6/32 antibody to HMW B27 indicates that this form also maintains some conformation of the peptide-binding groove.

However, it must be noted that the above result provides no information regarding the rest of the B27 molecule. Therefore, we decided to select antibodies against HMW B27 through the bio-panning of a phage-display antibody library, in order to use antibodies pre-selected for binding (through bio-panning) to conformationally probe the B27 chain in both its HMW and ternary complex forms. Phages displaying scFv antibodies [96 each from bio-panning of the Tomlinson I and J libraries (obtained from the MRC, UK)] were picked after three rounds of bio-panning and amplification. In a re-confirmation of the screening results obtained from this library, the bulk of these phages were observed to bind to HMW B27 through phage ELISA (Fig. S1, Supplementary data). After this, the phages were turned into phagemids through transformation of phages into bacterial cells designed to produce and secrete soluble scFv antibodies. The supernatants from 192 clones (96 each picked from screening of I and J libraries), which contain soluble scFv antibodies were then used to perform ELISA experiments for binding to HMW B27. A total of 24 clones were observed to contain soluble antibodies capable of binding to HMW B27 (the fact that this number is lower than 192 is not surprising because it is possible that the recognition of HMW B27 by the remaining antibodies in the phage ELISA experiments was dependent on the scFv antibodies remaining in fusion with the phage pIII coat protein, and dependent on packing of the folded antibody against the phage). Fig. 5 shows the ELISA assay for the 192 clones, in which 24 clones show binding values above the background.

Information concerning which of the extracts shows HMW-B27 binding was then re-confirmed through a fresh round of ELISA, and this information was then used to purify periplasmic extracts of antibodies from each of the 24 clones. Unfortunately, at this point, four of the clones could not be revived, and so we proceeded with soluble antibodies from only 20 clones. These antibodies were again tested for binding to HMW B27, as shown in Table 1, and all were re-confirmed to bind to the HMW form. Next, these antibodies were examined for binding to the ternary complex of the HLA B27 heavy chain with β_2m and nonameric peptide. It may be noted that the generation of this complex had been previously standardized for other experiments (see Materials and methods); here, the complex was bound to the microtitre plate in such a manner that the same amount of B27 heavy chain was effectively used for immobilization in each well, regardless of whether ELISA plates were prepared through coating of the HMW form of B27, or with the ternary complex (marked HMW or TC respectively in Table 1). Thus, discounting for problems of access to epitopes, the same amount of B27 heavy chain protein was used to cross-compare the binding of the scFv antibodies raised against HMW B27, to the ternary complex. Table 1 shows the relative binding of these 20 HMW B27-binding scFv antibodies to ternary complexes of B27 and to HMW B27. Column 5 in Table 1 shows the fold excess of ELISA signals of HMW B27 binding over that of binding of the ternary complex (TC) by the antibodies, as the ratio of the raw absorption signals obtained with the two forms. It may be noted that the negative control in which primary antibodies were not added during the procedure gave a value below 0.07 for both anti-HMW ELISA and anti-TC ELISA. The ratio of OD readings for the binding of scFv to HMW vs. that of binding to the ternary complex showed that 7 out of 20 clones exhibited preferential binding to HMW as determined by ratio of OD readings greater than 2 standard deviations (95% confidence interval) from a background average represented by the lowest 13 readings (3.707 ± 2.387). Therefore, in assessing fold excess of anti-HMW signals over corresponding anti-TC signals, we took any value up to 8.4:1 to be indicative of comparable binding of the two forms. Where fold excess values were almost 8.4:1, or more, we took this to indicate differential binding of the two forms.

Separately, the 20 scFv antibodies were also used in western blot experiments to examine whether they can bind to epitopes on the B27 heavy chain, following denaturation on SDS-PAGE and partial, or incomplete, denaturation. A total of 14 of these clones recognized the B27 heavy chain in the western blot (Fig. S2, Supplementary data).

Our analysis of the data used the following criteria, to obtain the results summarized in Table 1 in regard to content of native-like and non-native-like conformational epitopes:

(i) An antibody could bind, in principle, to either: (a) a continuous stretch of amino acid residues recognized in a particular folded conformation, or a non-folded, extended conformation (i.e., a sequence epitope), or to (b) a combination of two or more discontinuous stretches of amino acid residues coming together in the folded structure to give rise to a uniquely structured epitope that would certainly not be expected to exist in unfolded protein molecules (i.e., a conformational epitope), unlike a sequence epitope presented by

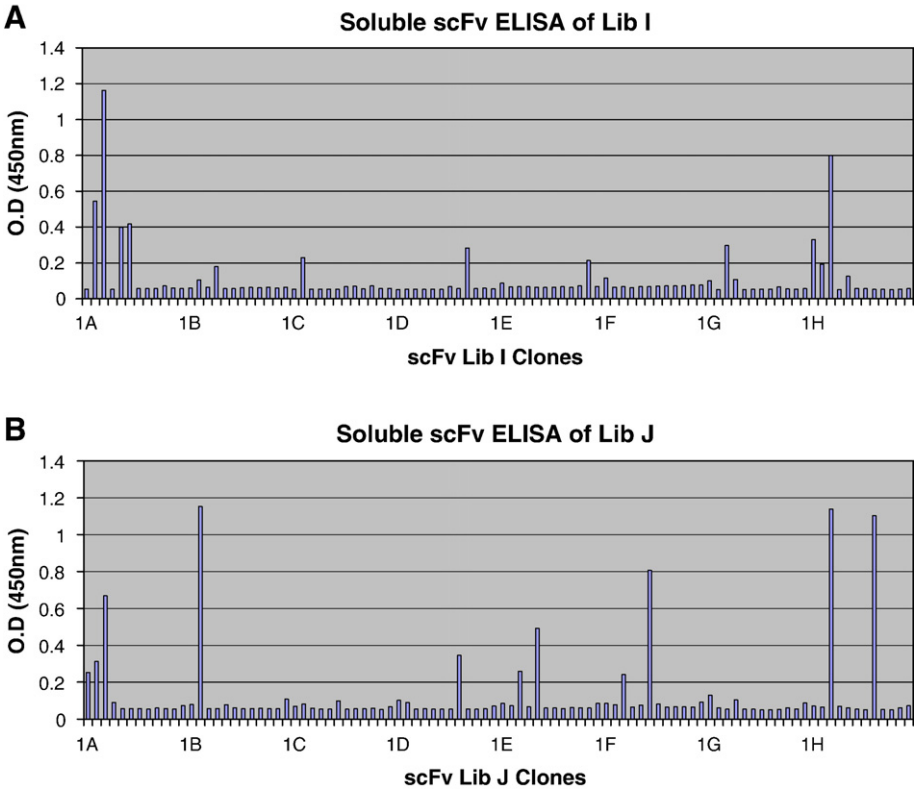

Fig. 5. Histograms showing ELISA values recorded with soluble scFvs (expressed in HB2151 cells) binding to coated HMW B27 in case of 96 clones from A) library I and B) library J respectively. The *y*-axis shows the optical density at 450 nm.

an unfolded, or partially-refolded protein. Although, of course, B27 polypeptides could potentially refold upon SDS removal during the western-blotting procedure to give rise to partially-folded, B27 chains showing up on western blots, we chose to assume the stringent criterion that antibodies showing binding on western blots bind mainly to sequence epitopes and not to conformational epitopes, as there is a higher likelihood of this being the case due to non-folding, or improper folding, of chains. According to this criterion, the antibodies that bind to sequence epitopes are I1H, I2A, I2C, I2H, I3A, I3H, I11E, J2A, J2B, J3E, J3F, J3H, J5E, and J6F (see data in Fig. S2, Supplementary data).

(ii) According to the reasoning given above, antibodies giving positive results in western blot, as well as comparable values of raw data in anti-HMW ELISA and anti-TC ELISA could be taken to bind to sequence epitopes that are exposed on the surface of both the HMW form, and the TC form. Ten antibodies from amongst those recognizing sequence epitopes were found to recognize both HMW and TC with a fold excess value of anti-HMW signals over anti-TC signals of less than 8.4:1. These were, respectively, I1H, I2A, I3A, I11E, J2A, J2B, J3F, J3H, J5E, and J6F (Table 1). Note that I2A showed a value of 8:1 (less than 8.4:1) and is technically also in this group.

(iii) Antibodies that gave positive results in western blot, and positive values in anti-HMW ELISA but not in anti-TC ELISA (i.e., with fold excess values of 8:4 or higher) were taken to bind to sequence epitopes exposed on the surface of only the HMW form, and not the TC form. These were, respectively, I2C, I2H, I3H and, most prominently, J3E. For the last of these, the fold excess was 39:1, indicating that this epitope is very definitely exposed on HMW B27 and buried in the B27 TC. It may be noted here that the reverse situation cannot obtain, i.e., there can be no sequence epitope-recognizing antibodies that give only anti-TC signals but no anti-HMW signals since, in the first place, the antibodies were selected for anti-HMW binding.

(iv) The antibodies that do not give positive western blot results, but give positive results in ELISA experiments, can be taken to bind to conformational epitopes. Of these, three antibodies, I4B, I5A and J1A were found to give signals of comparable intensity in anti-HMW and anti-TC ELISA experiments, indicating that they bind to a conformational epitope present on both HMW B27 and the B27 ternary complex.

(v) Three scFv antibodies, which gave negative results in western blot experiments, gave much higher intensity signals in anti-HMW ELISA experiments (i.e., a fold excess >8:4) than in anti-TC ELISA experiments. These were taken to bind to conformational epitopes displayed on HMW B27 that could be specific to HMW B27. These are the antibodies I3G, I9D and J8D.

3.5. Thermostability of structure

Fig. 2A showed that in the case of the HMW B27 chain there is no dramatic change in the CD signal strength at any wavelength in the range of 250–190 nm, upon increasing of temperature to 95 °C, which means that negligible thermal melting

of the protein occurs upon heating. The protein possesses nearly native-like levels of secondary structure even at this high temperature. A comparably high temperature of 98 °C was attained and used, for fluorescence measurements, with the help of fluorimetric and Peltier-based high temperature accessories on a Jasco CD spectropolarimeter. The λ_{max} of emission at 98 °C was also found to be 346 nm (Fig. 3), and not 353 nm as should have been the case if the HMW B27 had undergone thermal unfolding. This further confirms that the protein retains both secondary and tertiary structure at very high temperatures. In direct contrast, structural melting could clearly be observed to occur upon heating of trimeric complexes of HLA-B27 with β_2m and peptide, as seen in the data in Fig. 2B. When melting was monitored through observation of changes in ellipticity at 208 nm as a function of increasing temperature (data not shown), melting could be seen to have been initiated between 65 and 70 °C. Notably, previous studies [25,26] of the thermal melting of HLA-B27 complexes incorporating B2705 have also reported a melting temperature in the same range, corroborating our results with the complex.

3.6. Proteolytic evidence for a tight structure

The B27 chain is expected to generate exactly 33 fragments upon limit-digestion by trypsin, with the largest such fragment being 24 residues (~2600 Da) long. Therefore, any evidence indicating that proteolysis does not proceed to completion, or that a chain segment longer than this survives digestion, may be taken to be evidence of the existence of autonomously-stabilized sub-structures, since only formed structures could bury scissile peptide bonds away from trypsin. As can be seen in Fig. 6, many 'survivor' fragments remain upon overnight proteolysis, including some that are almost the size of lysozyme (14.4 kDa). Therefore, it may be concluded that one or more subdomain(s) of β_2m-free B27 survive limit-trypsinolysis after initial proteolytic release from HMW B27. One of these fragments, surviving the overnight digestion with trypsin (~12 kDa) was examined through N-terminal sequencing and found to have the residues F, I, T, V, G, Y at its N-terminus, which corresponds to the stretch initiating at residue 23 of the 362 residues-long B27 chain. Since this peptide is only 12 kDa in size, its C-terminus must lie some one hundred or more residues further away, from residue 23, towards the C-terminus of the B27 chain. Analysis of the trypsinolysis sites in the B27 chain suggests that the C-terminus of this fragment must constitute the sequence N-DAYDGK-C, i.e., that the survivor fragment must end with residue 122, since the sequence spanning the trypsinolysis sites at residue positions 22 and 122 of the B27 chain measures up to exactly 11,792 Da from sequence analysis, which is the closest to the size of 12 kDa. The closest competing sites at residue positions 112, and 146, respectively, would have resulted in fragment masses of 10,639 Da (considerably smaller than the observed size), and 14,446 Da (larger than the observed size). Thus we may conclude that the most structurally stable region of HMW B27 is the segment from residue 23 to residue 122, spanning the alpha 2 domain.

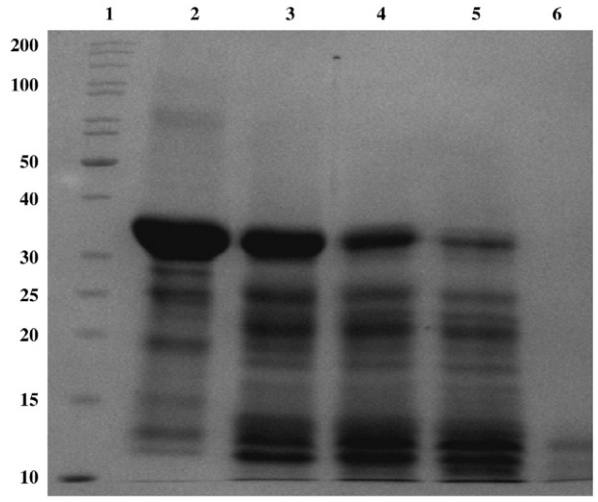

Fig. 6. Kinetic stability of HMW HLA-B27 to proteolytic cleavage by trypsin at 37 °C, using a trypsin:B27 ratio of approximately 1:6000. Lane 1 shows molecular weight markers. Lane 2 shows a trypsin-lacking control sample of the protein incubated overnight. Lanes 3, 4 and 5, respectively show products resulting from 1, 2 and 3 h of incubation respectively. Lane 6 shows products resulting from overnight incubation with trypsin. In lane 2, a certain amount of degradation has occurred in the control sample during overnight incubation without protease inhibitors.

3.7. Select scFv anti-HMW antibodies discriminate between ankylosing spondylitis (AS) patients and healthy volunteers

About twelve scFv antibodies (including those that bind specifically to HMW B27) were tested, in cell ELISA experiments conducted with human peripheral blood mononuclear cells (PBMCs) isolated from B27 negative, and B27 positive individuals (both from healthy individuals and those clinically diagnosed to have AS). These experiments allowed us to explore the presence of HMW-like forms of B27 on cell surfaces and simultaneously assess the importance of such forms to disease. With three of these antibodies significantly (3–5 fold) larger ELISA values were obtained with AS patients as compared to healthy B27 negative and B27 positive individuals (Fig. 7) whereas with the remaining antibodies comparable values were obtained for all three classes of samples (data not shown), except for I3H which, like the other antibodies, showed an insignificant difference between healthy B27 individuals and patients with ankylosing spondylitis, but at the same time showed a statistically significant difference between individuals who were negative for B27 and those positive for B27. This antibody, therefore, was used as a B27-reactive, but non-patient distinguishing control for the cell ELISA experiments, together with I3G, I9D and J2A. I3G and I9D, showed about 3–5 fold higher values with samples from AS patients than with other samples. The clone for the third HMW-specific antibody, J8D, showed poor expression levels at this stage of the study and could not be included in the cell ELISA experiments. Interestingly, J2A, also emerged as a diagnostic indicator of diseased status, suggesting that it may have been unfairly rejected as an HMW-specific antibody by our stringent criterion. It has been mentioned earlier that a western blot positive antibody could still involve a conformational epitope, since it is

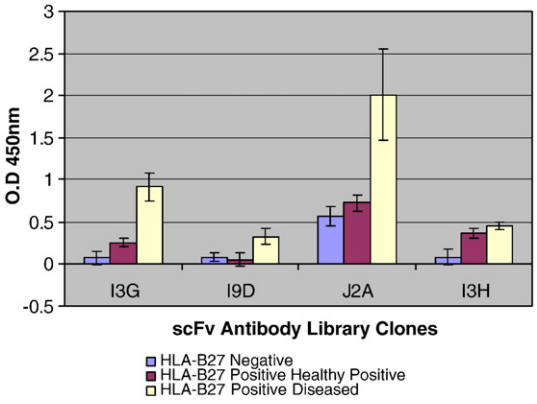

Fig. 7. Cell ELISA assay data with four scFv antibody clones, three of which showed differences between B27 negative, B27 positive but healthy, and B27 positive and diseased (ankylosing spondylitis) individuals. The fourth antibody, I3H, which distinguishes between B27-positive and B27-negative cells like the B27-reactive antibody, ME1, was used as a control.

conceivable for partial refolding to occur in the gel following SDS-removal, particularly given that the recombinant B27 chain in the gel would undergo refolding (of whatever sort) in the absence of β_2m. Thus we conclude that the J2A antibody too, like the I3G and I9D antibodies, binds to a conformational epitope specifically displayed on HMW B27, since its results in cell ELISA experiments with AS patients are a little over 2-fold higher than with other samples, similar to the differential binding of J2A to HMW B27 which is a little over 2-fold higher than to TC. However, in the discussion over the ability of J2A to distinguish AS patients, we must not forget that two scFv antibodies identified by us to bind specifically to HMW B27 (over 8.5 fold higher than to B27 in TC form) also similarly distinguish samples from B27 positive AS patients from other B27 positive and negative samples, to an even greater degree than J2A. This simultaneously suggests that HMW B27 is present on cell surfaces, and that it may also be associated with disease etiology. The statistical significance of these results was examined by applying the Student's unpaired *t*-test to the same 5 independent cell ELISA experiments for which mean and standard errors are shown in Fig. 7. The obtained p values describing the significance of differences in the binding of the four antibodies to healthy B27 individuals versus ankylosing spondylitis patients were 0.0014 (I9D), 0.0001 (I3G), 0.0008 (J2A), and 0.0988 (I3H). Similarly, the obtained p values describing the significance of differences in the binding of the four antibodies to B27-negative versus B27-positive individuals were 0.5 (I9D), 0.0026 (I3G), J2A (0.0448) and 0.0101 (I3H). Given that p values <0.05 are statistically significant, two conclusions can be made from these analyses: (i) I3H is an ME1-type antibody that is B27-reactive. (ii) That the differential binding of I9D, I3G and J2A to healthy B27-positive individuals and ankylosing spondylitis patients is statistically significant.

4. Discussion

Here we discuss our results in the light of what is already known about β_2m-free B27, and especially about HMW B27, in the published literature. As already mentioned, the bulk of the population that is found in the form of soluble protein – even in oxidation-favoring refolding environments – consists of HMW species of indeterminate association number [11]. The bulk of research attention thus far has, however, been paid to a population of homodimers which, as reported in this paper, is not seen in experiments that do not deliberately favor oxidation. Presumably, this lack of attention is because HMW-B27 has been assumed to consist of unstructured, or poorly structured, aggregates of little importance to disease mechanisms. Aggregates have traditionally been neglected as randomly-formed agglomerates of unfolded chains. However, as is well-known, they are increasingly being reported to be of importance to disease mechanisms. It is our case that the same may be applicable to HLA and disease.

Once a chain has engaged in HMW formation, it is likely that no scope remains for it to form a homodimer. In the earliest studies in which HMW-B27 was observed [11], it was found to display the mobility expected of a B27 monomer on SDS-PAGE only when samples were boiled in the presence of SDS under reducing conditions prior to electrophoresis. When reducing agents ordinarily used for electrophoresis were deliberately excluded from the sample loading buffer (prior to boiling of samples for SDS-PAGE), the HMW species did not dissociate to generate the expected SDS-bound monomeric B27 species during electrophoresis. A possible rationalization of this result is that unfolding of the associated B27 chains was occurring upon boiling in the presence of SDS, but that the dissociation of such chains into SDS-bound monomers was not occurring, because such dissociation was precluded (mainly by disulfide bonds that failed to be reduced). This indicates, of course, that disulfide bonds do exist in the HMW species and we do not contest this. However, very importantly, when cysteine 67 was replaced by serine through genetic manipulation [11], the minority population of homodimers could be no longer detected. The entire population could be seen to have transformed into HMW B27. This suggests two very important insights, namely: (i) that the formation of homodimers *requires* the formation of disulfide bonds involving Cys67, and (ii) that the formation of the HMW species *does not require* the formation of disulfides involving Cys67, even though such disulfides are clearly formed. The only conceivable structural-biochemical rationalization of this observation, for the moment, stems from what we have proposed in our recent hypothesis [19]: namely that noncovalent as well as covalent interactions exist in both the homodimer and the HMW species, but that disulfides play a more crucial role in stabilizing homodimers, and only an incidental role in stabilizing HMW species. Only when Cys67–Cys67 disulfide bonds form, do the reciprocally bound chains in a homodimer become sufficiently stabilized. When Cys67 is mutated to Ser67, there aren't enough interactions available to prevent dissociation of homodimers. Therefore, all molecules form the more (autonomously) stable HMW species that does not depend on Cys67–Cys67 disulfides for its stability. As a consequence, Ser67 mutants form only HMW species, and no homodimers. Such species are amenable to being unfolded and dissociated upon being boiled in the presence of SDS (even in the absence of reducing agents) because they have no disulfide bonds that need to be reduced prior to dissociation. This conclusion gets additional support

from our findings reported in this paper that wild-type HMW species do not unfold, or dissociate, when disulfides are reduced by 5 mM DTT, because there is now neither SDS nor high temperature available to break noncovalent interactions, and cause chain unfolding. In addition, electrophoretic experiments reported in this paper which include also the differential treatment of boiling, or lack of boiling, of samples reveal that DTT alone does not manage to completely dissociate HMW B27 and that accompanying denaturing influences are required in the form of either heat, or the presence of SDS. Therefore, it would appear that HMW B27 is principally stabilized by noncovalent interactions amongst structured B27 chains, with disulfide bonding involving Cys67 being merely incidental to the formation of this species.

In this paper, we have firstly provided evidence suggesting that this form of B27 contains alpha 1 and alpha 2 chains folded and assembled correctly enough to generate conditions for the immune recognition of a conformational epitope recognized by the w6/32 antibody. A sizeable fraction of this region is also shown to resist internal proteolysis by trypsin. Notably, the α1 and α2 domains assemble to give rise to the peptide-binding cleft of any Class I MHC molecule. Therefore, our results suggest that HMW B27 may contain peptide-binding clefts. Certainly, the structural content of HMW B27 and the extreme stability of this form of B27, taken together with the w6/32 binding data, indicate that B27 chains within HMW B27 are folded, and suggest that the domains of this molecule may be assembled into a native-like structural format.

The data with the scFv antibodies clearly demonstrates that sections of HMW B27 are native-like in structural content, whereas other sections are non-native-like. We identified at least three antibodies that bind to a conformational epitope on both HMW B27 and the B27 ternary complex, and at least ten antibodies that bind to sequence epitopes that are present on the surfaces of both forms and which may also conceivably be bound in the same conformation. These results suggest that there are significant commonalities between the structural organizations of the B27 chain in the two forms. At the same time, our results with the scFv antibodies demonstrate that there are also significant differences in the folding of the chain in the two forms. We identified at least four antibodies that bind clearly only to a sequence epitope, and three antibodies that bind to conformational epitopes presented only by the surface of HMW B27 and not by TC B27. No antibodies that bind only to conformational epitopes on B27 TC were identified because the phage display antibody libraries were screened for binding to HMW B27. We did not separately screen libraries for binding to B27 TC because of the potential infeasibilities of separating and distinguishing between antibodies binding to the B27 HC and β_2m.

Using cell ELISA experiments we show that two HMW conformation-specific antibodies (I3G and I9G) and one antibody that may be HMW conformation-specific even though it shows positive results with western blots (J2A) bind to cells of ankylosing spondylitis patients, but not to equivalent levels to cells from healthy B27 positive, or B27 negative individuals. These results hold great promise in further elucidation of the role of B27 misfolding to HMW forms in the etiology of disease.

If HMW B27 chains are indeed somewhat native-like, as suggested by these studies and if they possess assembled α1 and α2 domains forming peptide-binding clefts, are these clefts occupied, and if so with what? We have previously argued that an unstructured section of the B27 chain can separate away and bind to a B27 peptide-binding cleft to give rise to intermolecular cross-display. Such intermolecular swapping of sections has been reported in other soluble and insoluble HMW systems [27–30], and it is conceivable that HMW B27 also swaps other folded sections between neighboring molecules, i.e., sections that remain to be discovered (such as e.g., folded α2/α3 sub-domains). Certainly, such mechanisms could generate and stabilize multimers in a disulfide-independent fashion, and we have shown that the native-like B27 chains are indeed assembled into disulfide-independent multimers, even though such multimers do clearly contain intermolecular disulfides. We are thus inclined to suggest that since the bulk of the β_2m-free population consists of HMW B27, this species may be at least as important to the disease mechanism as the disulfide-bonded homodimer.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2007.10.005.

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