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Biochemical characterization of the human diabetes-associated HLA-DQ8 allelic product: similarity to the major histocompatibility complex class II I-A^{g7} protein of non-obese diabetic mice

The human HLA-DQ8 (A1*0301/B1*0302) allelic product manifests a strong association with insulin-dependent diabetes mellitus (IDDM). Previous biochemical studies of the major histocompatibility complex (MHC) class II I-A^{g7} protein of IDDM-prone non-obese diabetic mice produced controversial results. To better define the biochemical properties of IDDM-associated MHC class II molecules, we analyzed DQ8 proteins, in comparison to other DQ allelic products, by partially denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We now report that DQ8 proteins have a normal peptide occupancy and lifespan in cells. Similar to I-A^{g7}, DQ8 proteins formed only a minor fraction of SDS-stable complexes with peptides. Although this phenotype was not unique to DQ8, some DQ allelic products such as IDDM-protective DQ6 proteins were SDS resistant. The DQ9 allelic product, differing from DQ8 only at position (P) β57, was SDS stable, suggesting that non-Asp residues at β57 might decrease the SDS stability of DQ proteins. We identified a single peptide which specifically induced an SDS-stable conformation in DQ8 as well as in I-A^{g7} molecules. The residues at anchor P1 in this peptide were found to influence the SDS stability of both molecules. Together with our previous observation of similar binding motifs of I-A^{g7} and DQ8, these results demonstrate an overall biochemical similarity of mouse and human diabetes-associated MHC class II molecules. This similarity might contribute to a common immunological mechanism of IDDM in both species.

1 Introduction

Many autoimmune diseases are associated with certain MHC gene products [1]. In the case of the human autoimmune disease insulin-dependent diabetes mellitus (IDDM), a primary association is thought to exist with MHC class II HLA-DQ molecules [2, 3]. In particular, the strongest predisposition towards IDDM is conferred by the HLA-DQA1*0301/B1*0302 (DQ8) allele, while dominant protection from IDDM is associated with the DQA1*0102/B1*0602 (DQ6) allele [4]. Similar associations with the mouse homologs of HLA-DQ, the H2-A (I-A) molecules, are observed in the non-obese diabetic (NOD) mouse model of IDDM: the NOD I-A allele, I-A^{g7}, promotes the development of diabetes, while other MHC class II molecules were shown to exert a protective effect [5]. Although many polymorphic residues appear to influence the association of MHC class II alleles with IDDM [3], a salient feature of protective or predisposing alleles is the presence of Asp or non-Asp residues, respectively, at position 57 of the β chain [6].

The mechanism whereby MHC class II alleles influence the development of IDDM is not fully understood. Distinct peptide binding specificities of MHC class II alleles are likely to influence their association with IDDM and other autoimmune diseases [7]. Indeed, DQ8 and I-A^{g7} molecules manifest a rare preference for acidic residues at anchor P9 in the bound peptides, apparently due to the presence of non-Asp residues at β57 [8–11]. Interestingly, our recent analysis of the peptide binding motif of I-A^{g7} revealed its close similarity to the previously reported [8] binding motif of DQ8 [11].

However, other biochemical properties of IDDM-associated MHC class II products are less well characterized. Thus, it was reported that the I-A^{g7} molecule forms a negligible amount of SDS-stable complexes with peptides, has a reduced lifespan and is incapable of stable peptide binding [12]. Similarly, we found that only a small (but clearly detectable) proportion of I-A^{g7}-peptide complexes were stable in SDS; however, the lifespan and general peptide binding capacity of I-A^{g7} appeared normal [11]. Thus, it is not clear whether IDDM-associated MHC class II molecules are compromised in their peptide binding capacity. Recently, it was observed that purified DQ8 proteins were relatively unstable in the low concentration of SDS at neutral pH [13]; however, a systematic comparison between DQ8 and I-A^{g7} was not reported. To better define the biochemical properties of DQ8 and to compare them to those of I-A^{g7}, we here examined the SDS stability, peptide occupancy and lifespan of DQ8 in comparison to other HLA-DQ allelic products.

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Abbreviations: GAD: Glutamic acid decarboxylase **HSP60:** 60-kDa heat shock protein **IDDM:** Insulin-dependent diabetes mellitus **NOD:** Non-obese diabetic **P:** Position

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2 Materials and methods

2.1 Cells and reagents

The following homozygous EBV-transformed B cell lines were used: WT51, PRIESS, BSM (DQA1*0301/B1*0302, DQ8); H0104, PGF (DQA1*0102/B1*0602, DQ6); DKB (DQA1*0301/B1*0303, DQ9); TISI (DQA1*0501/B1*0301, DQ7); MANN (DQA1*0201/B1*0201, DQ2); HOM2 (DQA1*0101/B1*0501, DQ1); TOKUNAGA (DQA1*0103/B1*0601, DQ6w8). The cells were cultured at 37 °C in 5 % CO_2 in RPMI 1640 medium supplemented with 10 % FCS, 2 mM L-glutamine and antibiotics (complete RPMI 1640).

To facilitate comparison between HLA-DQ alleles and to rule out possible artifacts of the isolation procedure, DQ proteins were analyzed using broadly reactive mAb of different specificities. The mAb included L2, specific for the DQ α chain [14]; SPV-L3 [15], reactive with mature DQ$\alpha\beta$ dimers ([13] and B. R., unpublished); and HK19 [16], reactive with immature DQ$\alpha\beta$/invariant chain complexes (B. R., unpublished). Anti-rat TCR mAb R73 was used as an isotype-matched (IgG_1) control for mAb L2. HLA-DR proteins were isolated using mAb L243.

Peptides were synthesized by standard Fmoc chemistry using the AMS 422 synthesizer (Abimed, Langenfeld, Germany) and their quality was confirmed by HPLC. DQ8-binding peptides included the λ repressor 12–26 [8], the mouse CLIP peptide containing a 98Met $\rightarrow$ Glu substitution according to the DQ8 motif [8], and human glutamic acid decarboxylase (GAD) peptides 247-66 [8], 505-19 and 521-35 (two major GAD T cell epitopes in DQ8-transgenic mice; D. Altmann et al., submitted). The mouse 60-kDa heat shock protein (HSP60) peptide 166–185 (EEIAQVATISANGDKDIGNI), termed p12, is strongly immunogenic in NOD mice [17] and forms SDS-stable complexes with I-A^{g7} [11].

2.2 Cell labeling and immunoprecipitation

For metabolic labeling, cells were resuspended at 5×10^6/ml in methionine-free RPMI 1640 supplemented with 10 % dialyzed FCS, and incubated for 1 h at 37 °C. The cells were pulsed for 40 min with 100 µCi/ml [^{35}S]methionine, washed in PBS and incubated in complete RPMI 1640 at 37 °C for 4 h unless stated otherwise. For cell surface iodination, cells were washed, resuspended at 20–40 $\times$ 10^6/ml in PBS and incubated on ice for 5 min in the presence of 1 mCi/ml Na[^{125}I], 10 U/ml lactoperoxidase and H_2O_2 up to 50 µM. The cells were washed in 0.4 mg/ml tyrosine in PBS, and either snap-frozen or incubated in complete RPMI 1640 at 37 °C for the indicated periods of time. Total and SPV-L3-precipitable radioactivity was measured in a gamma-counter.

Labeled cells were lysed on ice in buffer A (50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 1 % NP40, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml aprotinin, 0.02 % NaN_3), centrifuged and precleared overnight with Protein A-Sepharose. The lysates were incubated with the appropriate mAb and the immune complexes were precipitated with protein A-Sepharose or, for mAb L2 (IgG_1), with protein A-Sepharose precoated with rabbit anti-mouse IgG_1 Ab (Pharmingen, San Diego, CA). The precipitates were washed, resuspended in a sample buffer containing 50 mM Tris/HCl, pH 6.8, 2 % SDS and 0.77 M 2-ME, and either boiled for 5 min or incubated for 40 min at room temperature as indicated. The eluted proteins were separated by SDS-PAGE in 10 % gels and analyzed by autoradiography.

2.3 The formation of SDS-stable MHC complexes with synthetic peptides

The formation of SDS-stable I-A^{g7}-peptide complexes was measured as described [11]. For HLA-DQ proteins, the same assay was used with modifications. Briefly, the cells were pulsed as described above with 200 µCi/ml [^{35}S]cysteine-methionine mix for 30 min and lysed in buffer A. Precleared lysates were adjusted to pH 5.0 with acetic acid and incubated for 1 h at 37 °C. This treatment resulted in the loss of the DQ-associated invariant chain and in a gain of the SPV-L3 epitope (not shown). The lysate was centrifuged for 5 min and diluted fivefold with buffer B (buffer A adjusted to pH 5.0 and containing 1.25 % octylglucoside instead of NP40). The resulting mixture was divided into aliquots and incubated for 4 h at 37 °C in the presence of the test peptides (200 µM). These conditions were previously found to enhance the dissociation of the invariant chain CLIP peptide from MHC class II molecules and to promote the formation of SDS-stable dimers [18]. After additional centrifugation and neutralization, HLA-DQ proteins were isolated with mAb SPV-L3 and analyzed by SDS-PAGE without boiling.

3 Results

To exploit the biochemical properties of the IDDM-associated DQ8 allelic product, we analyzed DQ8 proteins by an established technique of immunoprecipitation followed by partially denaturing SDS-PAGE [19]. As a control, we studied DQ6 proteins, which are protective against IDDM. Fig. 1 shows that the majority of DQ6 molecules at the cell surface formed an SDS-resistant compact $\alpha\beta$ dimer (C) indicative of stable peptide binding. In contrast, the majority of DQ8 heterodimers (but not the control HLA-DR4 heterodimers from the same cells) dissociated into separate chains in the presence of SDS. Nevertheless, a small amount of the DQ8 C dimer could be consistently detected. Furthermore, the SDS-unstable DQ8 molecules appeared to contain bound peptides, represented by the labeled material of low molecular mass at the front (1–3 kDa as determined by SDS-PAGE in 15 % acrylamide gels; data not shown) [11, 20].

Similar differences in the SDS stability were observed when DQ allelic products were isolated using another broadly reactive mAb (Fig. 2). Furthermore, DQ8 molecules appeared equally SDS-unstable in three B cell lines of different origins, *i.e.* WT51, PRIESS (Fig. 2) and BSM (not shown). Similarly, DQ6 molecules from two cell lines, PGF (Fig. 2) and H0104 (not shown), were equally SDS stable. Thus, the SDS stability of HLA-DQ-peptide complexes appears to be a consistent property of each allele. The DQ8 molecules isolated from the PRIESS line were

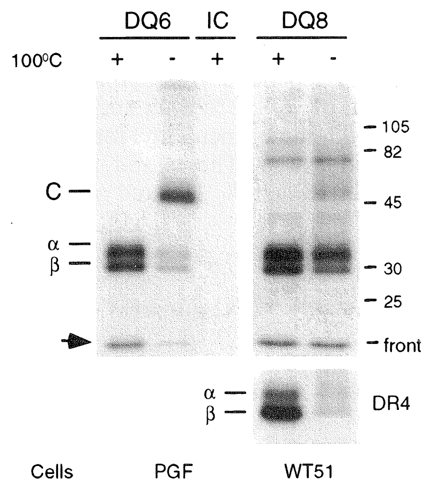

Figure 1. The stability and peptide occupancy of HLA-DQ8 molecules at the cell surface is shown. DQ8 and DQ6 proteins were isolated from membrane-iodinated B cell lines using mAb L2 and analyzed by SDS-PAGE with (+) or without (−) boiling. A negative control precipitation with an isotype-matched mAb (IC) is also shown. Isolated α and β chains as well as the SDS-resistant compact (C) αβ heterodimers are indicated. Low molecular mass material possibly representing MHC class II-bound peptides is indicated by the arrow. The positions of prestained protein standards, in kDa, are marked. The lower panel shows control HLA-DR4 molecules isolated from WT51 cells.

associated with a normal broad repertoire of natural peptide ligands [21]; therefore, DQ8 molecules are likely to contain bound peptides despite their relative instability.

We next analyzed several other DQ allelic products by partially denaturing SDS-PAGE (data not shown). We found that A1*0101/B1*0501 (DQ1), A1*0201/B1*0201 (DQ2) and A1*0501/B1*0301 (DQ7) allelic products were mostly SDS unstable, while A1*0103/B1*0601 (DQ6w8) proteins were SDS resistant. Thus, similar to I-A^{g7}, the SDS-unstable phenotype is not unique to DQ8 and can be observed in other HLA-DQ alleles.

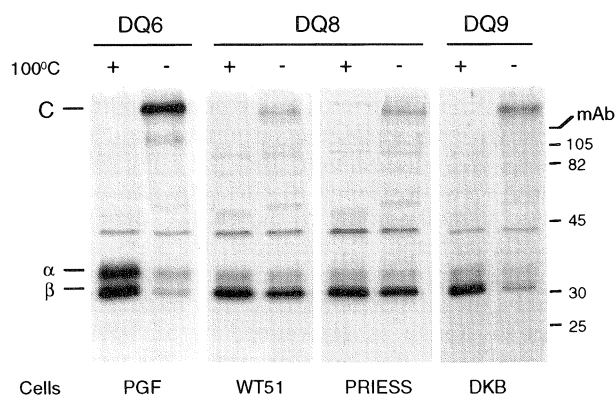

Figure 2. The formation of SDS-stable heterodimers by metabolically labeled mature HLA-DQ molecules is shown. DQ proteins were isolated from pulse-chased B cell lines using mAb SPV-L3. In contrast to the other mAb, SPV-L3 appeared in Coomassie-stained gels as a whole IgG molecule after partially denaturing SDS-PAGE (position indicated as mAb). The band (C) of about 200 kDa apparently represents the compact DQ dimer complexed with the mAb. Note the weak labeling of the DQ8/9 α chain (A1*0301), presumably because this chain contains only one Met residue compared to four Met residues in DQ6α and DQβ chains.

To test the influence of the Asp/non-Asp polymorphism at Pβ57 on the SDS stability of HLA-DQ molecules, we analyzed the DQA1*0301/B1*0303 (DQ9) allele. This allele is neutral to weakly protective against IDDM [4] and is identical to DQ8 except for the Ala → Asp substitution at β57. Fig. 2 shows that, in contrast to DQ8, DQ9 molecules were mostly SDS resistant. This result indicates that the amino acids at β57 may be involved in the regulation of SDS stability of HLA-DQ molecules.

To analyze the intracellular maturation and lifespan of DQ8 proteins, we performed pulse-chase experiments. Metabolic labeling revealed that newly synthesized DQ8 and DQ6 molecules were associated with the invariant chain, which was released during 1–4 h of chase to generate mature DQ heterodimers (data not shown). To estimate the dissociation of DQ-peptide complexes in living cells, we isolated DQ8 and DQ6 proteins at different time points after cell surface iodination. Fig. 3 shows that both DQ8 and DQ6 proteins persisted in cells for an equally long time characteristic of MHC class II molecules [20]. Indeed, anti-DQ-precipitable radioactivity after 24 h of chase was about 50 % of the radioactivity initially precipitable for both proteins, while the total cell-associated radioactivity decreased to <1 % (not shown). Notably, the SDS-stable and SDS-unstable fractions of DQ8 were equally long-lived (Fig. 3), suggesting that both fractions are occupied with stably bound endogenous peptides. Thus, DQ8 proteins appear to maintain a normally long lifespan in cells despite their relative SDS instability. These data also suggest that SDS-stable and SDS-unstable natural MHC class II-peptide complexes have similar dissociation rates.

To analyze the peptide binding capacity of DQ8 in more detail, we tested several known DQ8-binding peptides for their ability to form SDS-stable complexes with DQ8. Fig. 4 shows that most of the tested peptides failed to induce an SDS-stable dimer. However, we could identify

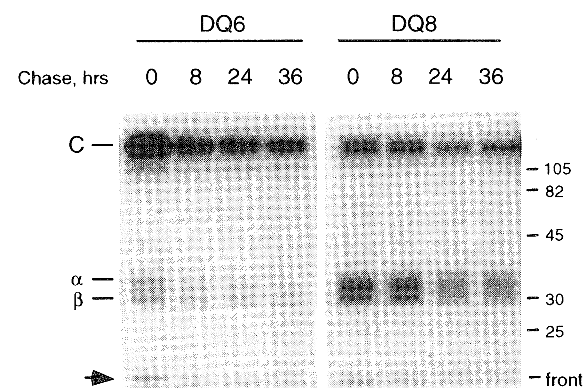

Figure 3. The lifespan of HLA-DQ8 molecules in cells is shown. WT51 (DQ8) and PGF (DQ6) cells were iodinated and incubated at 37 °C for the indicated periods of time. HLA-DQ molecules were isolated using mAb SPV-L3 and analyzed by SDS-PAGE without boiling. C marks a compact DQ dimer (see Fig. 2). The arrow marks DQ-associated peptides (see Fig. 1).

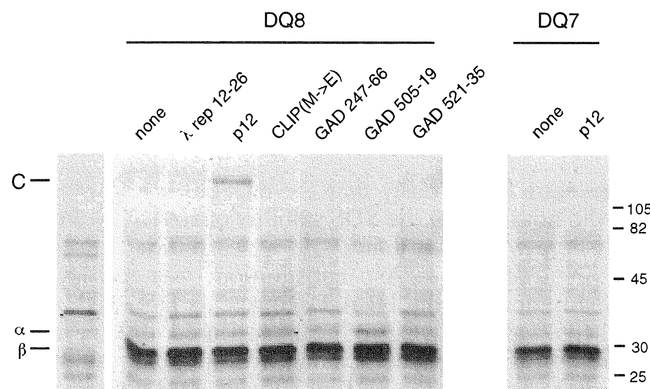

Figure 4. Peptide-induced formation of SDS-stable DQ8 dimers. The lysates of pulse-labeled WT51 (DQ8) or TISI (DQ7) cells were incubated at acidic pH in the presence of the indicated peptides, and the DQ molecules were isolated using mAb SPV-L3. The samples were not boiled before SDS-PAGE. The left lane shows a control precipitation from HOM2 cells, which express DQ1 molecules not recognized by the mAb.

one HSP60-derived peptide, p12, which was capable of stabilizing DQ8 molecules in SDS. The formation of the SDS-stable complex with p12 was specific for DQ8, since it was not observed with another SDS-unstable allele, DQ7. Study of the mouse I-A^{g7} molecule showed that, out of ten high-affinity peptides tested, only p12 conferred SDS stability to I-A^{g7} ([11] and B. R., unpublished).

To investigate a molecular basis of the uniquely stable binding of p12 to DQ8 and I-A^{g7}, we designed truncation/substitution analogs of p12. Fig. 5 shows that the same core region of p12 conferred SDS stability to both I-A^{g7} and DQ8 (minimal sequences: AQVATISANGDK for I-A^{g7} and QVATISANGDK for DQ8), consistent with a perfect I-A^{g7}/DQ8 binding motif present in this epitope. To preserve the MHC binding affinity of p12, we introduced substitutions at positions flanking the predicted nonamer binding core, as well as at anchor P1, which accomodates multiple residues in both I-A^{g7} and DQ8 motifs [8, 11]. Indeed, all studied truncation/substitution variants of p12 showed comparable I-A^{g7} binding capacities in a competition binding assay [inhibitory dose ($ID_{50\%}$) of 2–8 μM; data not shown]. In contrast, the SDS stability of the p12-I-A^{g7} complex was strongly affected by the substitutions tested (Fig. 5). Thus, a rare combination of flanking and anchor residues in a peptide, in addition to the basic I-A^{g7} binding motif, appears to promote the formation of an SDS-resistant complex with I-A^{g7}.

The influence of the substitutions tested on the SDS stability of a p12-DQ8 complex was less pronounced. In contrast to I-A^{g7}, the formation of a stable complex with DQ8 was not affected by substitutions at the flanking residues of the p12 core region (Fig. 5). This could be accounted for either by a true structural difference between I-A^{g7} and DQ8, or by the apparently lower sensitivity of the DQ8 stabilization assay. Nevertheless, residues at P1 of p12 appeared to affect the SDS stability of both I-A^{g7} and DQ8. Thus, even a conservative Val → Ala substitution produced a detectable effect (increase for I-A^{g7} and decrease for DQ8), while a Val → Glu substitution consistently abolished SDS stability of the complex in both molecules. These data indicate that DQ8 also shows stringent requirements for the formation of SDS-stable peptide complexes; these requirements appear similar but not identical to those of I-A^{g7}.

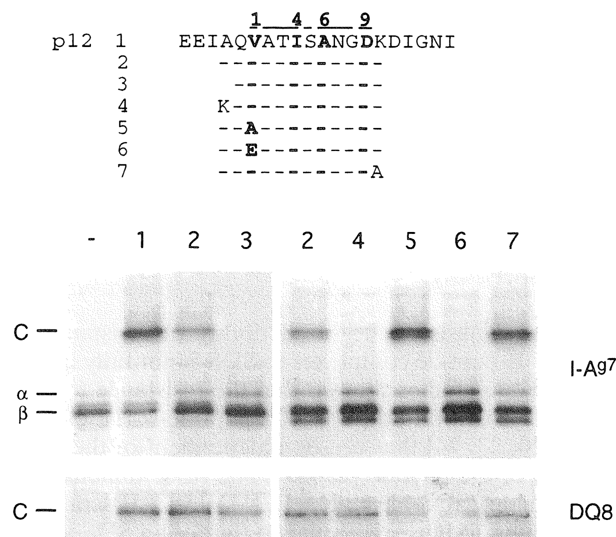

Figure 5. The formation of SDS-stable complexes by truncation/substitution variants of p12 is shown. The stabilization of I-A^{g7} was measured as described previously [11]. The stabilization of DQ8 was assayed as in Fig. 4; shown are the upper parts of gels (above 100 kDa). The truncation/substitution analogs of p12 are listed at the top; the predicted major anchor positions of the I-A^{g7}/DQ8 binding motif are numbered and highlighted in bold. The Gln → Ala substitution in p12 did not affect the SDS stability of the complex with I-A^{g7} (not shown).

4 Discussion

The present study was aimed at further defining the biochemical properties of MHC class II allelic products associated with IDDM. It was recently reported that the mouse I-A^{g7} molecule has a reduced lifespan and cannot form stable complexes with peptides [12]. However, we [11] and others [22] were unable to detect a fault in the peptide binding capacity of I-A^{g7}. Similarly, we show here that IDDM-predisposing DQ8 molecules maintain a normal lifespan in cells, appear to contain stably bound endogenous peptides and are intrinsically capable of forming SDS-stable complexes with peptides. These data further indicate that diabetes-associated MHC class II allelic products are not defective in their ability to stably bind peptides.

The I-A^{g7} molecule manifests a rare (but not unique) instability in SDS [11, 12]. Recently, it was reported that purified DQ8 (DQ3.2), after preincubation at neutral pH, was less stable in a low concentration of SDS than DQ3.1 (A1*0301/B1*0301) [13]; however, the unconventional assay used in that study complicated the comparison with I-A^{g7}. Here we demonstrate that, similar to I-A^{g7}, DQ8 proteins form only a minor but clearly detectable fraction of SDS-resistant complexes with peptides. Again, this instability was not unique to DQ8, as it could be observed in several other DQ alleles showing weak positive (DQ1) or unclear (DQ2, DQ7) association with IDDM [4]. It

should be noted, however, that the products of alleles protecting from IDDM, such as DQ6 and DQ6w8 [4], were SDS-resistant. It is unlikely that a straightforward correlation exists between SDS stability and the association with IDDM of MHC class II products; for example, an SDS-unstable β57Asp molecule, DQ7, was observed. However, one could speculate that an SDS-unstable or SDS-stable phenotype might be characteristic of MHC class II products conferring strong susceptibility or protection from IDDM, respectively.

The structural basis of the susceptibility or resistance of MHC class II-peptide complexes to SDS is not fully understood. Previous studies showed that the resistance to SDS is a distinct structural property of a peptide-MHC class II complex [20, 23, 24] that can be independent of the binding affinity of the peptide [25–27]. The three-dimensional structure of an SDS-unstable HLA-DR3-CLIP complex [28] was found to be very similar to that of an SDS-stable HLA-DR1-HA peptide complex [29], except for a small conformational change in the α-helical region of the β chain. In agreement with other reports, we here show that flanking regions [23, 25] and/or anchor P1 [26, 27] appear to influence the SDS stability without changing the binding affinity of the peptide-MHC complex. Thus, the SDS stability is likely to reflect a distinct conformation of a peptide-MHC class II complex, which depends on many specific residues in a peptide and can be rarely adopted in "SDS-unstable" proteins such as I-A^{g7} or DQ8. Importantly, stability in SDS appears to be a consistent polymorphic feature of MHC class II molecules, especially of the I-A [11, 12, 19] and DQ (this study) family, and thus reflects an intrinsic structural feature of each MHC class II allelic product. Therefore, the parameter of SDS stability appears useful for the comparative biochemical analysis of MHC class II products.

An important question is whether differential SDS stability of MHC class II molecules has a functional correlate. It was reported that SDS-unstable MHC class II-peptide complexes have a shorter lifespan in cells than SDS-stable complexes [30]; however, the peptide variants examined in that study varied greatly in their MHC class II binding affinity [23], and the behavior of synthetic peptides might be different from that of natural MHC class II ligands. Indeed, we observed that SDS-unstable I-A^{g7} [11] and DQ8 (this study) molecules have a normally long lifespan in cells, as would be expected for stable MHC class II-peptide complexes that have passed "quality control" by HLA-DM dimer molecules [31]. We have so far been unable to detect differences in the function *in vivo* of MHC class II-peptide complexes forming SDS-stable or SDS-unstable complexes with I-A^{g7}, using the p12 variants substituted at P1 (peptides 5 and 6 in Fig. 5) (B. R., unpublished). Both p12 variants persisted in living splenic APC for a comparably long time (>12 h) as revealed by T cell proliferation assays. When tested for immunogenicity in either CFA or IFA, both peptides induced equally strong regional and systemic T cell responses with an identical Th0 cytokine profile in NOD mice. Moreover, both peptides were equally tolerogenic when injected i.v. in soluble form. Thus, SDS-stable and SDS-unstable MHC class II-peptide complexes appear to be grossly equal in their immunogenicity and tolerogenicity. Hence, there is no evidence to indicate that the differential SDS stability of MHC class II proteins might influence their association with IDDM. At this point, we can only say that SDS stability can serve to probe structural features of MHC class II molecules, and that I-A^{g7} and DQ8 manifest an overlap in this regard.

It was shown previously that the β57Ser → Asp substitution did not increase the SDS stability of I-A^{g7} [12]. In contrast, here we report that DQ9, which differs from DQ8 only in a β57Ala → Asp substitution, was resistant to SDS. In addition, the HLA-DQ proteins DQ8 (β57Ala), DQ1 (β57Val) and DQ2 (β57Ala) appeared SDS unstable. Therefore, the presence of small hydrophobic residues at β57 might decrease the SDS stability of HLA-DQ-peptide complexes, possibly due to the abrogation of an interchain β57Asp-α76Arg salt bridge [32]. Conversely, an SDS-unstable β57Asp molecule (DQ7) was identified, suggesting that additional residues might regulate the SDS stability in the presence of β57Asp. These observations suggest that the Asp/non-Asp polymorphism at Pβ57 might determine not only the binding specificity, but also the overall conformation of MHC class II molecules.

Our analysis of the I-A^{g7} molecule [11] revealed a peptide binding motif very similar to the reported motif of DQ8 [8], suggesting that the same diabetogenic self-peptides might be presented by both molecules. Here we demonstrate that both proteins are SDS unstable and can be uniquely stabilized by a single peptide out of many peptides tested. Indeed, our preliminary analysis of the sequence requirements for the stabilization in SDS suggests that these requirements might be similar, but not necessarily identical, in both I-A^{g7} and DQ8. The biochemical similarity shown here between the I-A^{g7} and the DQ8 molecules, along with their similar binding motifs, might contribute a molecular foundation for the strikingly similar immunological expression of IDDM in mice and humans [33]. Our observations further underscore the relevance of immunological studies in NOD mice to human diabetes.

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