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Soluble HLA-DQ2 expressed in S2 cells copurifies with a high affinity insect cell derived protein

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Abstract We here describe that soluble HLA-DQ2 (sDQ2) molecules, when expressed in *Drosophila melanogaster* S2 insect cells without a covalently tethered peptide, associate tightly with the *D. melanogaster* calcium binding protein DCB-45. The interaction between the proteins is stable in S2 cell culture and during affinity purification, which is done at high salt concentrations and pH 11.5. After affinity purification, the sDQ2/DCB-45 complex exists in substantial quantities next to a small amount of free heterodimeric sDQ2 and large amounts of aggregated sDQ2 free of DCB-45. Motivated by the stable complex formation and our interest in the development of reagents which inhibit HLA-DQ2 peptide binding, we have further characterized the sDQ2/DCB-45 interaction. Several lines of evidence indicate that an N-terminal fragment of DCB-45 is involved in the interaction with the peptide binding groove of sDQ2. Further mapping of this fragment of 54 residues identified a pentadecapeptide with high affinity for sDQ2 which may serve as a lead compound for the design of HLA-DQ2 blockers.

Keywords HLA · sDQ2 · DCB-45 · S2 · Protein interactions · Peptide binding

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Introduction

HLA-DQ2 (DQA1*0501/DQB1*0201) is associated with several immune-mediated disorders including celiac disease (Sollid et al. 1989; Thorsby and Ronningen 1993; Todd et al. 1987). In order to develop specific blockers as a potential future treatment for celiac disease and other HLA-DQ2 associated diseases, there is a need to define high affinity ligands for HLA-DQ2. The development of such blockers will be facilitated by the production of recombinant HLA-DQ2 that can be made in high quantities and utilized for binding studies. On this background, we have produced recombinant, soluble HLA-DQ2 (sDQ2) molecules in either stably transfected S2 cells or in baculovirus infected Sf9 cells. Interestingly, when expressing sDQ2 in S2 cells without a covalently bound peptide that occupies the binding groove, we obtained remarkably high amounts of stable molecules. Further analysis of these sDQ2 molecules revealed that a major proportion of the molecules copurifies with the *Drosophila melanogaster* calcium binding protein DCB-45, a homolog to the *D. melanogaster* supercoiling factor (SCF; Kobayashi et al. 1998). Given the apparent ability of the protein to stabilize sDQ2, we hypothesized that it could contain a peptide which binds efficiently to the binding groove of sDQ2 and which could function as a lead peptide for the development of HLA-DQ2 blockers. Here, we report on the identification and characterization of the DCB-45 sequence which mediates binding to sDQ2. Next to characterizing the interaction between sDQ2 and DCB-45, our observations are of general relevance for expression of recombinant major histocompatibility complex (MHC) class II molecules in S2 cells.

Materials and methods

HLA expression and purification

Water soluble HLA-DQ2 (DQA1*0501/DQB1*0201) was expressed in a *D. melanogaster* S2 cell line by cotransfection of three vectors: a pMtal vector with the sequence encoding for the extracellular part of the DQα-chain fused to the Fos zipper, a pMtal vector with the sequence encoding the extracellular part of the DQβ-chain fused to the Jun zipper, and a pCoHYGRO resistance vector (Paulsen et al., unpublished). The construct did not include a sequence for a high affinity peptide ligand tethered to the β-chain. The stably transfected S2 cells were grown in 1L cell spin flasks at 22°C in serum-free media (Insect-XPRESS™, BioWhittaker, Walkersville, MD, USA), containing 300 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) and 25 μg/ml garamycin (Schering-Plough, Kenilworth, NJ, USA). The pMtal vectors contain a metallothionein promoter and the production of sDQ2 was induced by 100 mM $CuSO_4$ over 3 days. From cell culture supernatants, the sDQ2 molecules were affinity-purified like previously described (Quarsten et al. 2001). HLA-DM molecules were produced as soluble molecules in transfected S2 cells (kind gift of Elizabeth Mellins) and purified by FLAG-tag immunoaffinity chromatography and size exclusion chromatography as described (Sloan et al. 1995). Detergent-solubilized HLA-DQ2 (DQA1*0501/DQB1*0201; EBV-DQ2) molecules were purified from Epstein–Barr virus-transformed B lymphoblastoid cell lines as previously described (Johansen et al. 1994). The protein concentration of the various HLA molecules was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA).

Size exclusion chromatography and gel electrophoresis

Preparative size exclusion chromatography was performed on an Äkta purifier system (Amersham Biosciences Corp., Piscataway, NJ, USA) using a Superdex 200 10/300 GL column (Amersham Bioscience). Proteins were separated by isocratic elution (flow rate 0.75 ml/min) using phosphate buffered saline (PBS; pH 7.3) and monitored at 280 nm. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 6–12% polyacrylamide gels. Samples were reduced by heating in Laemmli buffer containing 1% β-mercaptoethanol (95°C, 5 min). Proteins were stained by Coomassie Blue.

Trypsin treatment

For protein identification by peptide mass fingerprinting, protein bands were excised from the Coomassie Blue stained gel and in-gel digested as previously described (Fleckenstein et al. 2004). For the acetylation of primary amino groups, the gel pieces were incubated in 3% acetanhydride/0.1 M $NaHCO_3$ pH 8.4 for 2 h at room temperature, followed by several washing steps and tryptic digestion. Partial digestion of the sDQ2/DCB-45 complex in solution was performed with *N*-tosyl L-phenylalanyl chloromethyl ketone (TPCK) trypsin agarose beads (Pierce). For 24 μg protein, 4.8 μl bead suspension in 0.1 M NH_4HCO_3 buffer in a total volume of 22.8 μl was used. The samples were incubated for 17 min at 37°C under rotation. For size exclusion chromatography, the beads were removed by filtration.

Mass spectrometry

Matrix-assisted laser desorption–ionization time-of-flight mass spectra (MALDI-TOF MS) were acquired on a MALDI-TOF/TOF instrument (Ultraflex II, Bruker Daltonics, Bremen, Germany). Tryptic peptide mixtures and peptides eluted from sDQ2 were desalted and concentrated on Poros 20 R2 reverse-phase packing sorbent (Applied Biosystems, Foster City, CA, USA) packed in 20-μl GELoader tips (Eppendorf, Hamburg, Germany). Peptides were eluted onto a stainless steel target plate using 70% acetonitrile, containing 0.1% trifluoroacetic acid (TFA) and 10 g/l α-cyano-4-hydroxycinnamic acid. Synthetic peptides were also analyzed using α-cyano-4-hydroxycinnamic acid, but then samples were applied as a dried droplet.

Peptide synthesis

Peptides with a length of 11–20 amino acid residues were synthesized on Rink amid methylbenzhydrylamine-resin using Fmoc/2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) chemistry and a pipetting robot (Syro I, MultiSynTech, Bochum, Germany). Coupling was performed with a tenfold molar excess of each Fmoc-L-amino acid and HBTU and a 15-fold excess of *N*,*N*-diisopropylethylamine. Piperidine (20% in dimethylformamide) was used for Fmoc deprotection and 96% TFA containing 2% triisopropylsilane and 2% water were used for side chain deprotection and cleavage from the resin. The identity was confirmed by MALDI-TOF MS, and the purity was determined by analytical reversed-phase high performance liquid chromatography (HPLC; Agilent 1100 system, Agilent Technologies, Santa Clara, CA, USA) using a Zorbax C18 column (Agilent Technologies). The 54 residue long peptide was synthesized on 10 mg NovaPEG Rink Amide resin LL (Novabiochem®, Merck KGaA, Darmstadt, Germany) with a low loading capacity of 0.16 mmol/g. The pseudoproline dipeptides Fmoc-Gly-(Hmb)Gly-OH (Novabiochem) in position 24 and Fmoc-Glu(OtBu)-Ser($\psi^{Me,Me}$pro)-OH (Novabiochem) in position

41 and 52 were used to substitute for Gly-Gly and Glu-Ser, respectively. The secondary amide bond of these amino acid surrogates is reversibly protected and cleaved during deprotection with 96% TFA. The first 30 cycles were run under standard conditions as described above. From cycle 30 on, all coupling steps were repeated and 30% piperidine in dimethylformamide for Fmoc deprotection was used. Side chain deprotection and cleavage from the resin was performed as described above. From the crude peptide, the observed TFA ester on the N-terminal Ser side chain was removed by treatment with 1 M NaOH for 30 min, followed by neutralization with equimolar amounts of HCl. The identity and purity were determined like described above.

Labeling of synthetic peptides

The HLA-DQ2 high affinity ligand P198 (KPLLIIAEDVE-GEY, *Mycobacterium bovis* 65 kDa Hsp 243–255Y) was either fluorescently labeled or radiolabeled. The radio-labeling with ^{125}I was done with the chloramine T method (Greenwood et al. 1963). Fluorescence labeling was done by N-terminally coupling of 5(6)-carboxyfluorescein (CF) using 5 equivalents CF and 6 equivalents *N*,*N*-diisopropyl-carbodiimide. The coupling step was repeated several times until a negative Kaisertest was obtained (Kaiser et al. 1970). The fluorescently labeled peptide was cleaved from the resin and analyzed as described above.

Peptide binding assays

Detergent-solubilized HLA-DQ2 (DQA1*0501/DQB1*0201; EBV-DQ2) at concentrations of 0.1–0.2 μM was incubated with the radioactively labeled P198 indicator peptide and synthetic peptides at various concentrations as described earlier (Johansen et al. 1994). The EBV-DQ2-peptide complexes were subsequently separated from unbound peptides by size exclusion chromatography in a spin column system as described (Buus et al. 1995). The radioactivity in the void volume and in the column material was counted by a γ-counter (Wallac, Turku, Finland) and IC50 values were calculated. sDQ2 (6.25 μM) or trypsin digested sDQ2 (6.25 μM) was incubated with CF-labeled P198 indicator peptide (10 μM). In some instances, soluble HLA-DM (sDM) was added in a concentration of 0.38 μM. The incubation was performed in a total volume of 13 μl of a citrate phosphate buffer (pH 5.3) at 37°C over 48 h. Next, 5 μl of each sample were transferred on a Zorbax GF 450 4.9×250-mm column and eluted with 150 mM PBS at a flow rate of 1 ml/min using an Agilent 1100 HPLC system. The UV signal (214 nm) and the fluorescence signal (Ex. 490 nm, Em. 520 nm) were monitored. Peptide binding to sDQ2 was quantified by measuring the area under the curve (AUC) of the fluorescence signal for peaks with a maximum between 9.5 and 9.7 min (corresponding to sDQ2 with bound indicator peptide). The mean values from at least two independent experiments were plotted.

Results

Characterization of the sDQ2 preparation by SDS-PAGE and size exclusion chromatography

The affinity purified sDQ2 was analyzed by SDS-PAGE under reducing conditions. In addition to the bands of 35 and 25 kDa corresponding to the sDQ2 α- and β-chains, respectively, a protein with an approximate molecular weight of 50 kDa was observed (Fig. 1A). In size exclusion chromatography under nonreducing conditions, the same sample eluted in three peaks, which were fractionated for further analysis by SDS-PAGE (Fig. 1B,C). As the first peak eluted with the void volume, we consider it to represent aggregates of sDQ2 with a size larger than 600 kDa. SDS-PAGE analysis of this fraction (Fig. 1C, lanes a and e) showed the presence of sDQ2 α- and β-chains, but no band at 50 kDa. In the second fraction (Fig. 1C, lanes b and f), the 50-kDa protein was detected with similar intensity as the sDQ2 α- and β-chains. Peak 3 mainly contains sDQ2 α- and β-chain (Fig. 1C, lane c). The small amount of the 50-kDa protein can be explained by the overlap of peak 2 and 3 and the small amounts of peak 2 present in fraction 3. The amount of protein applied to each lane of the SDS-PAGE was calculated by combining the known amount of total protein subjected to size exclusion chromatography and by determination of the peak areas for each of the fractions 1–3. This was 2.2 μg for each of the lanes a–c. For the lanes e and f, 29 μg of protein of the fractions 1 and 2, respectively, was loaded in each lane. The protein bands in the lanes a and e (corresponding to fraction 1) appeared weaker compared to the protein bands detected in the lanes b and f (corresponding to fraction 2), although identical amount of protein should have been loaded. This might be due to aggregation of sDQ2 in fraction 1, leading to an incomplete transfer into the gel.

Identification of the 50-kDa protein by peptide mass fingerprinting

In order to identify the nature of the 50-kDa contamination, the protein was subjected to tryptic in-gel digestion. The digest was analyzed on a MALDI-TOF/TOF mass spectrometer and a MASCOT search reported the identification of a *D. melanogaster*-derived SCF (accession number Q9W0H8_DROME). Acetylation prior to digestion and analysis by MALDI-TOF/TOF MS identified the tryptic

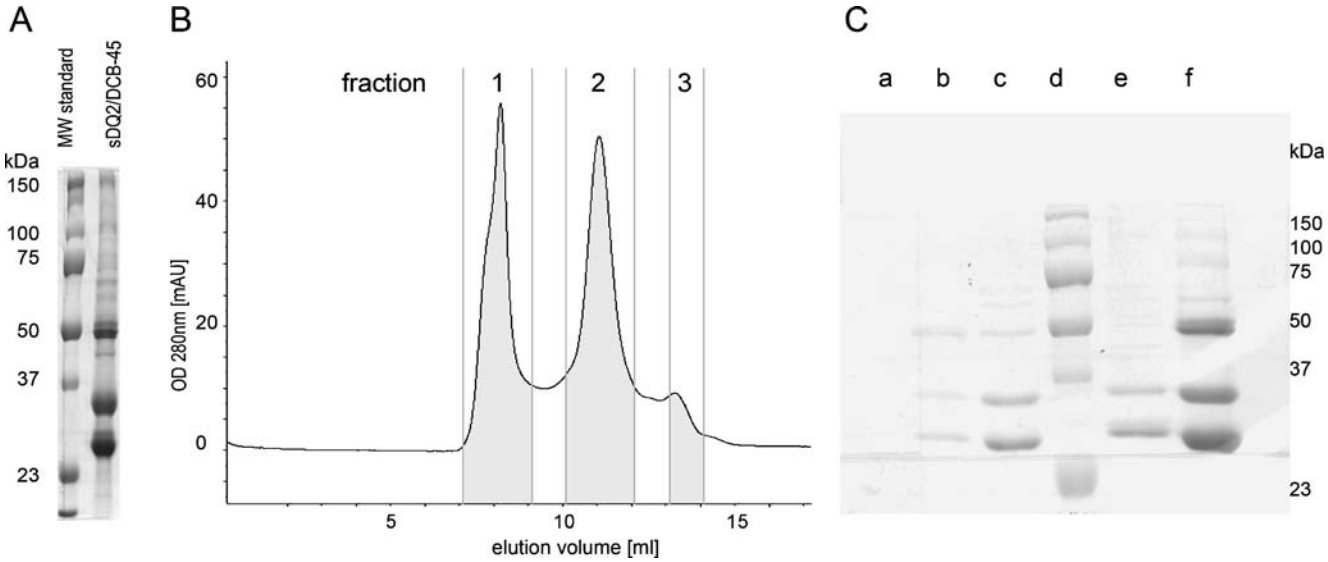

Fig. 1 Analysis of sDQ2 produced in S2 insect cells by reducing SDS-PAGE (**A**, **C**) and size exclusion chromatography (**B**). **A** SDS-PAGE of the purified sDQ2 preparation. **B** Analysis and fractionation of the produced sDQ2 by size exclusion chromatography. **C** The three fractions obtained in **B** were reanalyzed by SDS-PAGE. *Lanes a–c*, *fractions 1*, *2* and *3*, respectively (2.2-μg protein loaded); *lane d*, molecular weight standard (BIO-RAD, precision plus protein™ standard); *lane e and f*, *fraction 1* and *2*, respectively (29 μg protein loaded). Amounts of protein loaded onto the gel were calculated from the known amount of total protein subjected to size exclusion chromatography and determination of the peak area

peptide SSIPEELPHNPLEHDPLHPR as the N terminus of the protein. The observed 50-kDa protein therefore represents a N-terminally truncated derivate of the *D. melanogaster* calcium binding protein (DCB-45), which is described as an elongated homolog of SCF (Kobayashi et al. 1998). The identified protein is highly acidic with a p*I* of 4.27.

Limited digestion of sDQ2/DCB-45 complexes

To investigate the interaction between DCB-45 and sDQ2, we subjected the obtained sDQ2 preparation to limited tryptic digestion with immobilized TPCK trypsin. As evaluated by SDS-PAGE, a digestion time of 17 min at 37°C was found to be optimal to fully degrade DCB-45

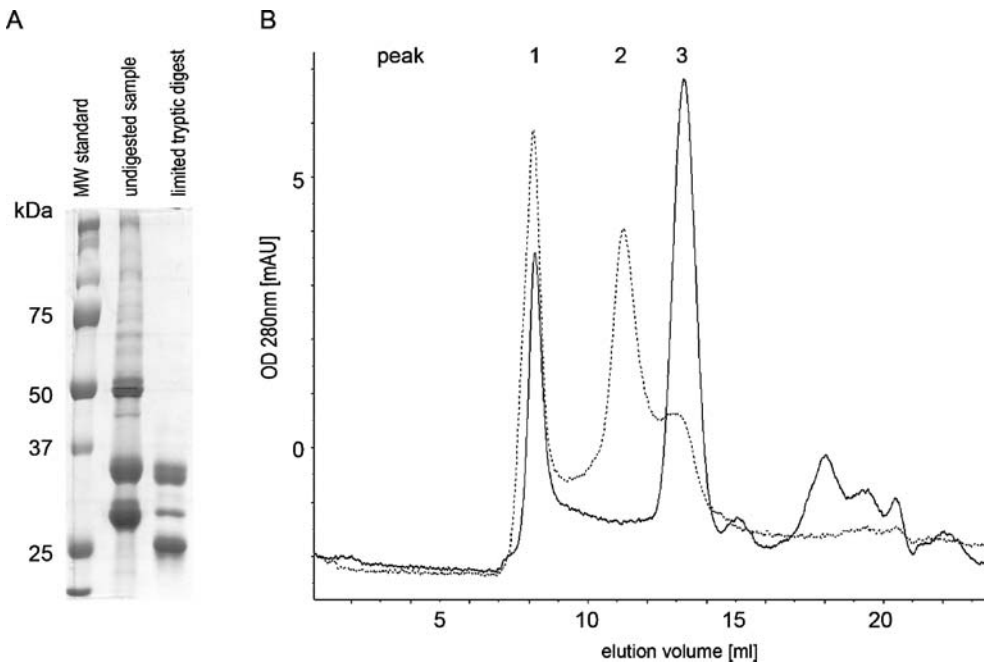

Fig. 2 Limited tryptic digestion of the purified sDQ2 preparation analyzed by SDS-PAGE (**A**) and size exclusion chromatography (**B**). **B** Overlay of the size exclusion chromatograms of the nondigested (*broken line*) and trypsin treated sDQ2 preparation (*full line*)

(Fig. 2A). Under these conditions, the sDQ2 α-chain remains intact whereas most of the β-chain undergoes a small truncation which does not interfere with formation of a stable and functional heterodimer (see below). Size exclusion chromatography of the same digested sample demonstrated a complete disappearance of the sDQ2/DCB-45 complex which eluted in peak 2 of the untreated sample (Fig. 2B). Notably, the limited proteolysis resulted in a significant increase of the signal intensity observed for peak 3. Following purification of peak 3, the sDQ2 in this preparation was found to be stable for at least 2 days when coincubated at 37°C not only with the high affinity HLA-DQ2 binding peptide P198 but also without adding peptide for at least 24 h at 4°C (data not shown). This suggests that sDQ2 in the partly digested sample remains as a stable heterodimer with an occupied and thereby stabilized peptide binding groove. Peak 3 of the trypsin-treated sDQ2 preparation (Fig. 2B) was concentrated over a 10-kDa spin filter (Vivaspin), washed with PBS, and treated with 0.1% TFA to elute ligands occupying the peptide binding groove. Analysis of the eluted peptides by MALDI-TOF/TOF MS identified four tryptic peptides which were all derived from the 54 N-terminal amino acid sequence of DCB-45 (Fig. 3). These peptides share a 23 amino acid long core sequence (HFDGGEHNAQFD HEAFLGPDESK), suggesting the interaction site is within this region. Besides the DCB-45-derived peptides, we found low signal intensities for several other peptides in the MALDI-TOF spectrum. One of these peptides could be identified as STEFSEDLLDEDLDLSDIDENEEEFLR, and this fragment is derived from the *D. melanogaster* protein RH45818p (Fig. 3).

Peptide binding ability of sDQ2/DCB-45 before and after limited tryptic digestion

The sDQ2/DCB-45 complex (i.e., peak 2 in Fig. 2B, broken line) was purified, and its peptide binding ability was analyzed using the fluorescence-labeled HLA-DQ2 high affinity ligand P198. Peptide binding to sDQ2/DCB-45 was found to be poor, and also limited tryptic digestion of the complex could only slightly increase binding of P198 (Fig. 4). In the presence of HLA-DM, however, binding of P198 was remarkably increased, both for the trypsin treated and untreated sDQ2/DCB-45.

Identification of the HLA-DQ2 peptide binding frame and binding affinity of DCB-45-derived peptides

To further investigate whether the interaction between DCB-45 and sDQ2 is involving the peptide binding groove, we synthesized five 20-mer peptides with overlapping sequences which covered the 54 amino acid long N-terminal DCB-45 sequence (Table 1). The peptide binding affinity was tested in the competition assay using the ^{125}I-labeled indicator peptide P198 and EBV-DQ2. In this assay, the highest affinity was found for the 20-mer PRHFDGGEHNAQFDHEAFLG. To identify the exact binding frame, three 15-mer peptides covering this 20-mer region were synthesized and tested. The best binding sequence was found to be HNAQFDHEAFLGPDE with an IC50 of 0.13 μM. The truncated peptide HNAQFD-HEAFL also bound with a good affinity (0.77 μM), suggesting AQFDHEAFL as the 9-mer core region mediating HLA-DQ2 binding (Table 1). The IC50 value of the

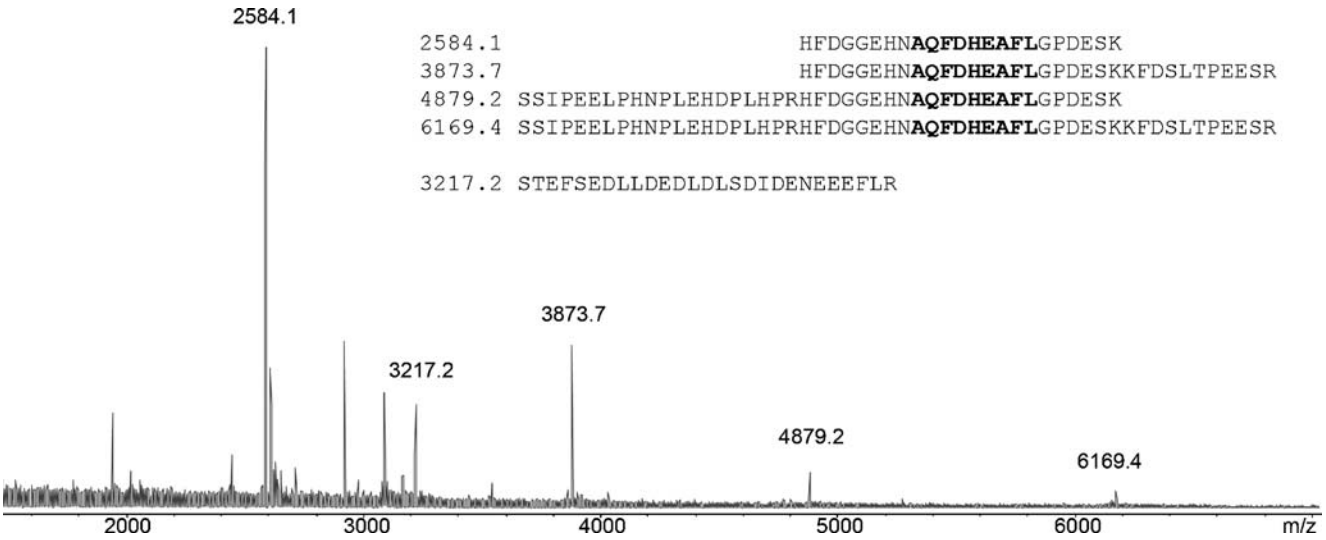

Fig. 3 MALDI-TOF mass spectrum of acid-eluted peptides after trypsin treatment and fractionation of the heterodimeric sDQ2 fraction (peak 3; Fig. 2B). The sequences of the five peptides assigned by their *m*/*z* values were determined by MALDI-TOF/TOF MS and are given in the insert. Four of the identified peptides derive from DCB-45. The 9-mer, suggested as the core region interacting with the HLA-DQ2 peptide binding groove, is shown in *bold face*

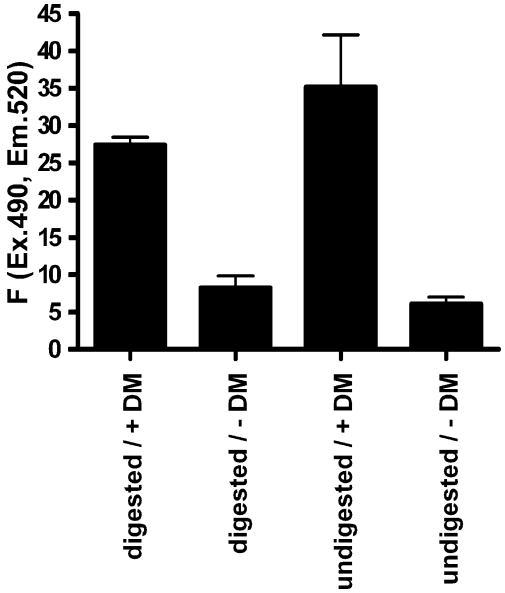

Fig. 4 Binding of the HLA-DQ2 high affinity ligand P198 to sDQ2/DCB-45. Carboxyfluorescein-labeled P198 was incubated with the purified sDQ2/DCB-45 complex with or without prior limited tryptic digestion and in the presence or absence of soluble HLA-DM (sDM). Binding was quantified in a gel-filtration assay and is given by the fluorescence signal (AUC) measured for the sDQ2/DCB-45 complex (undigested sample) or sDQ2 after limited digestion (digested sample). The signals were observed at an elution time of 9.5–9.7 min. Values were obtained from at least two independent experiments; mean and standard errors are shown

54-mer is higher than that of the best binding pentadecapeptide (Table 1), what can obviously not be explained by the slightly lower purity of the 54-mer (72%). Likely, a different secondary structure of the longer peptide hinders the interaction with the peptide binding groove resulting in the observed lower binding affinity.

Peptide binding specificity of sDQ2 compared to EBV-DQ2

The α-I-gliadin epitope and analogs were tested for binding to sDQ2 and EBV-DQ2. For both DQ2 molecules, the calculated relative binding capacity and the ranking of the measured IC50 values for the set of peptides was found to be nearly identical. This strongly indicates that the peptide binding specificity of sDQ2 matches that of EBV-DQ2 (Fig. 5).

Discussion

In this study, we show that soluble HLA-DQ2 molecules without a covalently tethered peptide ligand copurify with the *D. melanogaster*-derived protein DCB-45, when expressed in S2 insect cells. We identified the fragment of DCB-45 which mediates the strong interaction with sDQ2 and obtained evidence indicating that a part of this fragment is binding to the sDQ2 binding groove with high affinity. The sDQ2 stabilizing effect of the DCB-45 protein motivated us to unravel the basis for this interaction as

Table 1 Binding of synthetic peptides to EBV-DQ2 given as IC50 values

Peptides	IC50 [μM]
SSIPEELPHNPLEHDPLHPRHFDGGEHN**AQFDHEAFL**GPDESKKFDSLTPEESR	0.8[a]
SSIPEELPHNPLEHDPLHPR	51.1[b]
NPLEHDPLHPRHFDGGEHN**A**	>167[b]
PRHFDGGEHN**AQFDHEAFL**G	0.56[b]
N**AQFDHEAFL**GPDESKKFDS	1.09[b]
LGPDESKKFDSLTPEESRRR	7.76[b]
LHPRHFDGGEHN**AQF**	>167[b]
FDGGEHN**AQFDHEAF**	4.64[b]
HN**AQFDHEAFL**GPDE	0.13[b]
HN**AQFDHEAFL**	0.77[b]
KPLL**IIAEDVGEY**	0.09[b]
QLQ**PFPQPELPY**	1.1[a]
PQPELPYPQPQLPY	7.4[a]
KPLL**IIAEDVGEY**	0.06[a]

IC50 is the peptide concentration required to give 50% inhibition of the binding of the indicator peptide (P198). The IC50 values for the peptides presented in this table were analyzed in two separate sets of experiments. The results from one of three consistent titrations are given. As a reference peptide, P198 (KPLLIIAEDVGEY) was included. The HLA-DQ2 binding frames are indicated in bold face

[a] In the first set of experiments, the binding affinity of the 54-mer peptide (72% pure) compared to gliadin-derived peptides P1269 (HLA-DQ2-α-I epitope; QLQPFPQPELPY), P1274 (HLA-DQ2-α-II epitope PQPELPYPQPQLPY) and indicator peptide P198 was analyzed

[b] In the second set of experiments, the binding of overlapping synthetic peptides spanning the 54 amino acid long N-terminal region of DCB-45 was analyzed

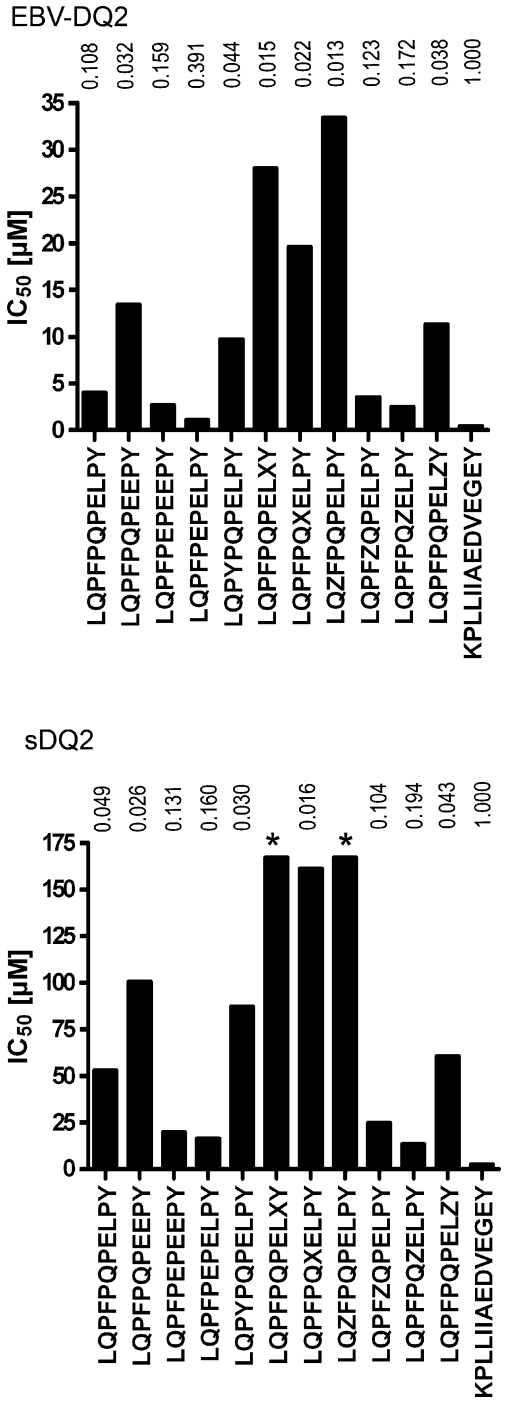

Fig. 5 Peptide binding specificity of sDQ2 compared to EBV-DQ2. sDQ2 (1 μM) and EBV-DQ2 (0.3 μM) was incubated with the radioactively labeled indicator peptide P198 (KPLLIIAEDVEGEY) and titrations of competitor peptides. The HLA-DQ2-α-I gliadin epitope (LQPFPQPELPY) and analogs were used. The *bars* show the IC50 values (in μM). The relative binding capacity for each peptide is given and was calculated as the ratio of the IC50 of the reference peptide P198 to the IC50 of the test peptide. The *Z* in the amino acid sequence denotes norvaline, *X* denotes 4-hydroxyproline. All peptides marked with an *asterisk* (*) have an IC50 value higher than 167 μM and the exact value could not be determined

part of an effort to design compounds that can block HLA-DQ2-mediated antigen presentation.

Expression of HLA class II molecules in insect cells is a commonly used method for the production of recombinant molecules (De Wall et al. 2006; Stern and Wiley 1992; Wallny et al. 1995). Initially, DR1 and other MHC class II molecules were expressed without a covalently tethered peptide as so called "empty" class II molecules (Stern and Wiley 1992). The expression of recombinant molecules with a high affinity ligand covalently tethered to the N terminus of the β-chain (Crawford et al. 1998), however, gave improved expression and stable molecules and this strategy thus developed as a preferred expression modality. Our early work to express sDQ2 without a covalently tethered peptide in the S2 cell system gave unusually high protein yields and stable molecules what is in contrast to a poor expression yield and unstable molecules obtained with similar baculoviral constructs in Sf9 cells (Quarsten et al., unpublished). When analyzing and comparing peptide binding specificity of these sDQ2 molecules with detergent-solubilized HLA-DQ2 purified from EBV-transformed B cells (EBV-DQ2), we found the same peptide binding preferences but approximately a tenfold increase of the IC50 values for each peptide when using the sDQ2 molecules (Fig. 5).

In mammalian antigen presenting cells the assembly of HLA class II α- and β-chains is dependent on the invariant chain (Ii) protein. In the ER, Ii is protecting and stabilizing the class II binding groove with its class II-associated invariant chain peptide (CLIP) sequence, and the Ii is directing the class II molecules to the late endosomes where HLA-DM catalyzes the exchange of CLIP for antigenic peptides (Busch et al. 2005; Cresswell 1994). In S2 cells, this mechanism for sDQ2 assembly and loading is missing. One might thus expect that the unoccupied amphiphilic peptide binding groove is rendering the class II molecules unstable and prone to aggregation (Rabinowitz et al. 1998; Vogt et al. 1997). For this reason, it has been argued that so-called "empty" class II molecules are not truly empty but filled with loosely bound peptides. We thus wanted to characterize sDQ2 molecules produced in S2 cells in more detail.

By SDS-PAGE analysis under reducing conditions, we observed a strong band at 50 kDa in addition to the bands of the sDQ2 α- and β-chains. A similar type of contamination has not been observed with other soluble HLA molecules produced in baculovirus infected Sf9 insect cells, nor in the wild type EBV-DQ2 produced by EBV-transformed B cells. This contaminating protein was identified as the *D. melanogaster*-derived DCB-45 protein and our findings suggest a tight interaction between sDQ2 and DCB-45. The complex does not dissociate under the condition of high pH and high salt concentrations used for elution from the

antibody affinity column (pH 11.5 and 2 M Tris), and treatment at low pH resulted in precipitation rather in dissociation of the two proteins. In contrast, the sDQ2 molecules found in the large aggregates (>600 kDa) apparently are not associated with DCB-45, suggesting that sDQ2 is unstable in the absence of DCB-45. The sDQ2/DCB-45 interaction thus likely explains the observed high expression yield of peptide-receptive sDQ2.

DCB-45 is a highly acidic protein (p*I* 4.27) and HLA-DQ2 has a strong preference for binding negatively charged residues in several of its anchor positions (Kim et al. 2004). We therefore investigated whether the sDQ2/DCB-45 binding is also involving the peptide binding groove of sDQ2. The characterization of such a strong interaction could be helpful to design new lead structures for high affinity HLA-DQ2 blockers. Such reagents have been discussed in the treatment of celiac disease to inhibit HLA-DQ2-rectricted presentation of gluten derived epitopes (Bergseng et al. 2005; Xia et al. 2006, 2007).

In the presence of HLA-DM, binding of the known HLA-DQ2 ligand P198 to the isolated sDQ2/DCB-45 complex increased. The same effect of HLA-DM was observed after limited proteolysis of this complex leading to almost exclusive degradation of DCB-45 and a markedly increase of heterodimeric sDQ2 (Fig. 2). Analysis by size exclusion chromatography showed that this fraction was stable. Interestingly, DCB-45-derived peptides could be released at low pH and were found to originate from the N-terminal 54-mer (Fig. 3). As these findings strongly indicate a specific interaction of this region with the sDQ2 peptide binding groove, a more detailed mapping was performed. Using detergent-solubilized EBV-DQ2, the pentadecapeptide HNAQFDHEAFLGPDE was found as the best binder with an affinity similar to that of the P198 ligand. The data also suggest the sequence AQFDHEAFL as the 9-mer core region with negative charges in positions P4 and P6 and a large hydrophobic residue in position P9 that are favorable for interaction with the sDQ2 peptide binding groove. This sequence contains alanine in position 1, which is not expected to be favorable due to its small size and the preference that HLA-DQ2 has for bulky hydrophobic anchor residues at this position (van de Wal et al. 1996; Vartdal et al. 1996). Notably, peptide binding to HLA-DQ2 is characterized by contributions from anchor residues in position 1, 4, 6, 7, and 9 (Kim et al. 2004). This is in contrast to peptide binding to HLA-DR molecules which has a dominant contribution of the P1 anchor (Jardetzky et al. 1990; Stern et al. 1994). Given that the peptide AQFDHEAFL has optimal anchor residues at positions P4 (D), P6 (E), and P9 (L), an alanine residue at P1 with no repulsive effect may well be compatible with the high affinity binding of this ligand. Moreover, in silico searching for possible binding registers based on binding studies with peptide libraries (Jüse et al., unpublished) gave no better candidates for high affinity HLA-DQ2 ligands within the 54-mer region. The DCB-45-derived 15-mer HNAQFDHEAFLGPDE is a good HLA-DQ2 binder, and we found that it is binding to HLA-DQ2 with a higher affinity than the HLA-DQ2-α-I-gliadin peptide that is an immunodominant epitope of celiac lesion derived CD4 T cells. Work parallel to this study (Xia et al. 2007) has demonstrated that even higher binding affinity than that of the DCB-45-derived pentadecapeptide as well as high proteolytic stability are required to obtain HLA-DQ2 blockers that effectively prevent activation of gliadin reactive T cells. Several chemical modifications that improve HLA-DQ2 binding affinity and proteolytic resistance would thus be required to make the DCB-45-derived ligand becoming an effective HLA-DQ2 blocker.

The observations presented in this paper are relevant for anyone aiming to express MHC class II molecules in S2 cells. DCB-45 is influencing the availability of free and functional peptide binding site of recombinant sDQ2, resulting in a decreased peptide binding capacity of the expressed molecules. Our findings illustrate that de novo interaction of recombinant MHC class II molecules with proteins derived from the expression system can occur. Prior to large scale use in peptide binding studies, such MHC class II molecules should be carefully characterized and compared with respect to peptide binding specificity and capacity to the wild type molecules, which are, e.g., purified from Epstein–Barr virus-transformed B lymphoblastoid cell lines.

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