A novel domain on HLA-DRβ chain regulates the chaperone role of the invariant chain

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Accepted 20 July 2006
Journal of Cell Science 119, 4207-4214 Published by The Company of Biologists 2006
doi:10.1242/jcs.03177

Summary

The human lymphocyte antigen (HLA) class II region encodes highly polymorphic peptide receptors, which associate in the ER to the chaperone invariant chain (Ii). Ii facilitates assembly of class II subunits to functional peptide receptors. We searched for a conserved structure on HLA-DR polypeptides that mediates contact to a previously identified proline-rich class-II-binding sequence of Ii. Major histocompatibility complex (MHC) class II β chain sequences exhibit two conserved tryptophan residues separated by 22 amino acids. Inspection of this motif in the X-ray structure of DR3 showed TrpTyr residues in the vicinity of the Ii-derived fragment CLIP. Five DRβ mutants were produced. Mutation at Tyr123, Trp153 and Asp152 residues abolished interaction to the proline-rich sequence of Ii. All mutants formed heterodimers with DRα, were capable of binding an antigenic sequence and were expressed on the cell surface of transfected cells. In the presence of endogenous DRβ chain however, the TyrAspTrp mutant was not cell-surface exposed and did not co-isolate with Ii or DRα. The competition of the mutant with the endogenous DRβ for binding to DRα indicates that a structure on DRβ chain regulates assembly of DR subunits. Hence, the chaperone function of Ii is mediated through a conserved region on the β2 domain of class II.

Key words: MHC subunit assembly, Invariant chain

Introduction

MHC class II (MHCII) molecules are peptide receptors that present antigenic peptides on the cell surface of antigen-presenting cells to $CD4^+$ T cells. MHCII α and β subunits assemble in the ER with the chaperone invariant chain (Ii) (Anderson and Miller, 1992). Ii binds to the peptide-binding groove and promotes folding and transport of the αβ heterodimer (Amigorena et al., 1995). Assembly with Ii alters the conformation of the MHCII heterodimer (Verreck et al., 2001). This conformational change of MHCII molecules may initiate intracellular transport and subsequent maturation of the class II peptide receptors.

An additional role of Ii is to sort MHCII heterodimers to endocytic compartments (Bakke and Dobberstein, 1990). A signal sequence on the cytoplasmic tail of Ii guides MHCII molecules to endocytic vesicles, where internalized antigen is encountered (Bremnes et al., 1994). Upon release of Ii in MHCII vesicles, antigenic peptides are bound to the class II cleft (Roche and Cresswell, 1991). Mice with an interrupted Ii gene show reduced MHCII surface expression and are deficient in presenting antigen, which indicates that Ii plays an important role in the MHCII-processing pathway (Elliott et al., 1994; Viville et al., 1993). Investigation of proteolytic processing of the MHCII/Ii complex in endocytic vesicles revealed that after digestion, a fragment of Ii (class-II-associated Ii peptide or CLIP), remains bound to the MHCII cleft. CLIP is subsequently released by DM molecules from the MHCII heterodimer. The X-ray structure of HLA-DR3 with bound CLIP demonstrated that the Ii-derived peptide is lodged in the MHCII peptide-binding groove (Ghosh et al., 1995). CLIP binds like antigenic peptides to the MHCII cleft (Malcherek et al., 1995). Polymorphic residues in the α1β1 domain of MHCII heterodimers modulate the binding affinity to CLIP (Sette et al., 1995). Transfection of different combinations of MHCII allo- and isotypes into fibroblast cells revealed a varying efficiency of cell-surface expression (Sant et al., 1991). Later studies indicated that the CLIP segment in Ii regulates assembly and intracellular transport of the class II heterodimers (Romagnoli and Germain, 1994). The contact of Ii to polymorphic side chains in the class II groove suggests that varying degrees of Ii dependency occur during class II subunit assembly (Bikoff et al., 1995). However, to achieve a chaperone role of Ii for all MHCII allo- and isotypes, interaction of Ii to a conserved region of the MHCII heterodimer is required. Previously, it was shown that a proline-rich sequence of Ii stabilizes binding of Ii to the MHCII heterodimer (Stumptner and Benaroch, 1997). Amino acid residues 82-86 of Ii mediate promiscuous binding to the MHCII polypeptides (Siebenkotten et al., 1998). The interaction site of this proline-rich sequence of Ii on DR molecules has not been identified. The residues Pro82, Pro84, Pro85 and Pro87 of Ii are adjacent to the $\alpha_2\beta_2$ domain of DR and could interact to a conserved region of the class II polypeptides. We searched for a proline-binding structure on MHCII heterodimers.

In this study a conserved sequence on MHCII β chains was discovered, which contains two tryptophan residues that form in their three-dimensional structure parallel sheets with tyrosine residues. One pair of Trp and Tyr and an adjacent Asp residue on DRβ chain mediate contact to a proline-rich sequence of Ii. The interaction of Ii to this novel motif on

DRβ chain modulates the chaperone function of Ii for class II folding and assembly.

Results

Search for a conserved invariant-chain-binding domain on MHCIIβ chains

Previously in the sequence of Ii, a polyproline was identified that interacts with HLA-DR and stabilizes the heterodimer (Stumptner and Benaroch, 1997; Siebenkotten et al., 1998). An example of a proline-binding sequence is the WW domain, which is named after a pair of strictly conserved tryptophans that are separated by 22 aa residues (Chen and Sudol, 1995; Zarrinpar and Lim, 2000). In the search for a proline-binding sequence we found a highly conserved WW-like structure in the primary sequence of MHCIIβ chains of vertebrate species (Fig. 1A). The sequence contains two Trp residues separated by 22 aa residues and two conserved Tyr residues. The resolved crystal structure of DR molecules showed that despite the distant location of the Tyr and Trp residues in the primary sequence (Trp153 and Tyr123 or Trp131 and Tyr171), both Trp and Tyr pairs form pocket-like structures that resemble the pair of TrpTyr residues in WW domains (Fig. 1B) (Bork and Sudol, 1994). The three-dimensional structure of the WW-defined motif in the β2 domain of DRβ chain differs however from the WW domain (see Discussion). To distinguish the DRβ sequence from the WW domain we designated this novel motif as WWCII (WW class II). The X-ray crystal structure of DR3 with bound CLIP (aa 81-101) revealed that the N-terminus of the Ii-derived peptide, containing the proline-rich region, was resolved only at residue Pro87 (Ghosh et al., 1995). Fig. 1 shows that Pro87 of Ii is directed towards the pocket-like structure formed by the Trp153 and Tyr123 residues of the DRβ chain. It is possible that the aromatic surface-exposed residues Trp153 and Tyr123 arrange a binding site for adjacent residues of the proline-rich Ii sequence (aa 82-87). In addition, the position of the acidic Asp152 residue, which could interact with the basic Lys83 residue of Ii, is shown (Fig. 1B).

Mutation of the WWCII sequence of the DRβ chain

To identify residues important for interaction of DRβ chains with the proline-rich sequence of Ii, we introduced mutations into the β chain of the DR1 allotype. The DR1β chain was mutated at Tyr123, Trp153 or Asp152, at both Tyr123 Trp153 and at Tyr123, Trp153 and Asp152 residues (compare Fig. 1A with 1B). Asp152 was mutated to examine interaction of the acidic residue with basic Lys residues of Ii. The Tyr, Trp and Asp residues were changed to Ala. To examine whether the mutated β chains from DR1 form dimers with the non-polymorphic DRα chain, cDNAs encoding mutated β and α chains were transiently expressed in COS-7 cells. Cells were labeled for 30 minutes with [^{35}S]methionine and lysed with NP40. DR heterodimers were immunoprecipitated with I251. This mAb detects a conformation-sensitive epitope on the DRβ chain and binds when the DRαβ dimer is formed. Fig. 2A demonstrates isolation of the mutant DR molecules with I251 (lanes 1 to 5). For comparison, wild-type DR was separated in lane 6. The result in Fig. 2A indicates that all mutant DR1β chains exhibit extensive folding and dimerisation with the DRα chain.

To examine whether the introduced mutations affect the overall folding of the α1β1 domain of DR, we tested whether the MHCII groove formed by the mutated DR1 heterodimer is capable of binding antigenic sequences. We used a recombinant Ii chain where the complete MHCII-binding site, including the proline-rich sequence, was replaced by a DR1-specific sequence derived from a matrix protein from influenza

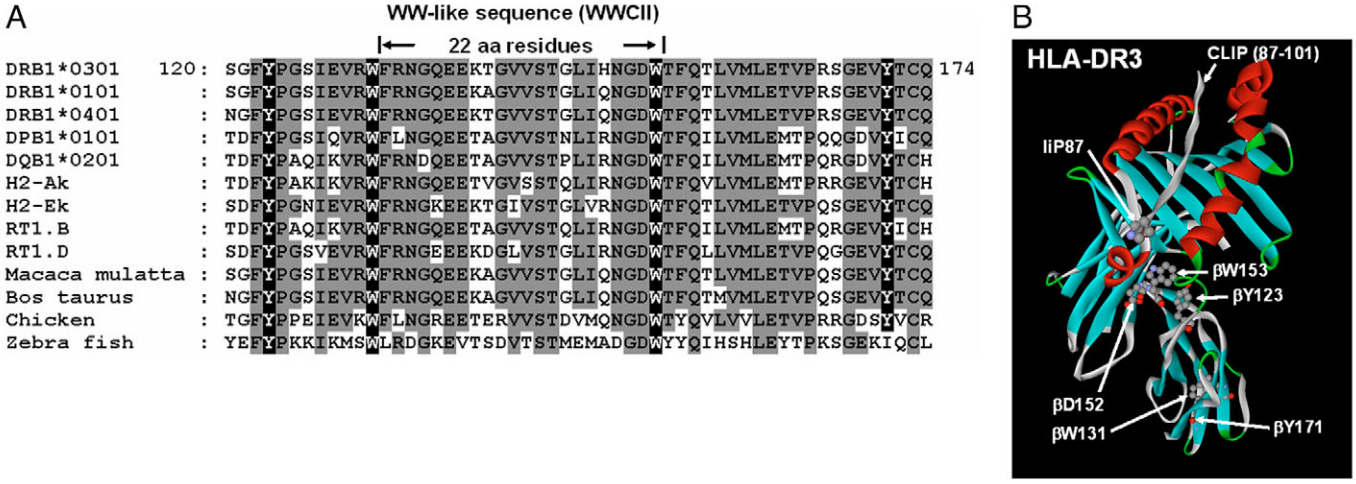

Fig. 1. A WW-like sequence on the DRβ chain. (A) Sequence alignment of MHCII β chain sequences. A list of sequences from HLA-D β allo- and isotypes and of MHCII β chain orthologs from several species is shown. The positions of conserved Y and W residues are labeled in black. Conserved amino acid residues are colored in gray. (B) A potential proline-binding sequence of the DRβ chain is adjacent to the proline-rich sequence of Ii. The X-ray structure of HLA-DR3 loaded with CLIP is shown. The YWD residues of WWCII are indicated (pdb accession 1A6A). Amino acid residues DRβW153 and DRβY123 form a hydrophobic cluster that is exposed to a potential position of the proline-rich region of Ii. Residues DRβW131 and DRβY171 form a cluster that is imprinted in the sequence of other members of the Ig super gene family. The P87 residue of CLIP is resolved in the X-ray structure. The X-ray structure was modified with Swiss-Deep view3.7 and visualized with Viewer Lite5.0 (right).

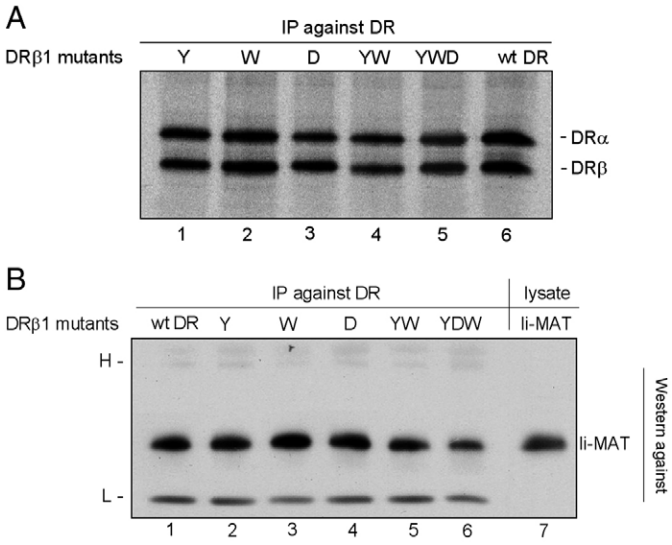

Fig. 2. Heterodimer formation and antigen binding of mutated DRβ chains. Point mutations were introduced into DR1β chain at residues Y123, D152 or W153 at Y123 and W153 and at Y123, D152 and W153, which were substituted by A. Mutant DR1 and wild-type DR1β chains were co-expressed with DRα in COS-7 cells. Residues Y, D and W, mutated to A are indicated on top. Cells were metabolically labeled with [^{35}S]methionine for 30 minutes. DR heterodimers were immunoprecipitated from cell lysates with mAb I251. Immunoprecipitates separated by SDS-PAGE were exposed to films for 24 hours. The positions of the DRα and DRβ bands are indicated at the right. (B) Binding of an antigenic influenza-virus-derived sequence (MAT) to DR1 mutants. A recombinant Ii (Ii-MAT), where the MHCII-binding sequence was replaced by a DR1-binding sequence derived from the matrix protein of influenza virus was co-expressed with mutant (lanes 2 to 6) and wild-type DR1β chains (lane 1), combined with DRα chain. DRβ chains with mutated Y, D and W residues are indicated on top of lanes 2 to 6. DR was immunoprecipitated with I251. Upon separation of the immunocomplexes by SDS-PAGE, Ii-MAT was immunoblotted with In-1 against Ii. Separation of Ii-MAT served as a reference for the recombinant Ii (lane 7). Heavy (H) and light (L) chains are indicated on the left and the position of Ii-MAT is shown at the right.

virus (MAT). Binding of Ii-MAT to MHCII heterodimers occurs in the endoplasmic reticulum and does not depend on antigen processing or on the function of DM. Previously, we showed that Ii-MAT–DR1 complexes are stable in NP40, whereas Ii-MAT–DR4 complexes could not be detected, indicating that Ii-MAT binds allotype specifically to DR1 (Siebenkotten et al., 1998). COS-7 cell were transfected with DRα, with mutant or wild-type DRβ, and with IiMAT-encoding cDNAs. In Fig. 2B mutated DR1 heterodimers and wild-type DR1 were immunoprecipitated. The co-isolated Ii-MAT was detected by western blotting with a mAb specific for Ii. Note that all DR1 mutants co-isolate Ii-MAT. The result indicates that the peptide-binding cleft of the mutated DR1 heterodimer binds to an antigenic sequence, such as the MAT sequence. Fig. 2B (lane 6) suggests that binding of the TyrTrpAsp mutant DRβ to Ii is reduced compared with wtDR (lane 1), which could indicate some conformational change of the TyrTrpAsp mutated DRβ chain affecting the peptide binding groove.

Deletion of the proline-rich sequence of Ii inhibits interaction with WWCII on the DRβ chain

The five DR1 mutants Tyr123, Asp152, Trp153, Tyr123Trp153 and Tyr123Trp153Asp152, were tested for binding to full-length Ii. For comparison, co-isolation of full-length Ii with wild-type DRαβ dimers is shown in Fig. 3A (upper panel, lane 1). Fig. 3A (upper panel, lanes 4, 7, 10, 13 and 16) shows that immunoprecipitation of the mutant DR1 heterodimers co-isolates Ii, which was monitored by western blotting. Thus, Ii is capable of binding to dimers of mutant DR1β chains with DRα. To examine interaction of the proline-rich sequence of Ii with MHCII, we constructed recombinant Ii chains, where the MHCII-groove-binding site at aa 91-99 was mutated. In the Ii91-99 mutant, the groove-binding sequence of Ii was replaced by a sequence that contains no binding motif for DR1. This sequence was extended further in the Ii82-99 mutant to replace the proline-rich sequence. DR immunoprecipitates from COS-7 cells transfected with wild-type DR, with DR mutants and with Ii91-99 showed detection of the recombinant Ii (Fig. 3A, upper panel, lanes 2, 5, 8, 11 and 14), except for the TyrTrpAsp DRβ mutant (lane 17). DR heterodimers composed of DRα and TyrTrpAsp mutant DRβ bound wild-type Ii but not the mutant Ii91-99 that contains the proline-rich sequence and no MHCII-groove-binding sequence. This result indicates that Ii91-99 binds to DRαβ heterodimers and suggests that aa residues 82-90 of Ii are important for binding to the complex. The Ii82-99 mutant that contains no proline-rich sequence showed almost no binding to wild-type DR and no co-isolation with the mutant DR heterodimers was detected (Fig. 3A, lanes 3, 6, 9, 12, 15 and 18). Expression of the transfected polypeptides was examined by western blotting of cell lysates (lower part of Fig. 3A). Isolation of αβ dimers was achieved by using a conformation-sensitive mAb, which selectively immunoprecipitates DR heterodimers. To estimate the relative amount of Ii chain co-precipitated with DR, the band intensity of co-isolated Ii was compared with Ii expression in the corresponding cell lysate.

To verify the nature of binding of Ii to DR heterodimers, an additional allotype was examined. Tyr123Trp153Asp152 residues of DR3β were mutated to Ala123Ala153Ala152. The mutant DR3β chain was co-expressed with DRα and tested for binding to recombinant Ii chains. The DRα DR3β heterodimer with mutated TyrTrpAsp binds to wild-type Ii (Fig. 3B, lane 4), albeit with reduced strength, but shows no binding to mutants Ii91-99 (Fig. 3B, lane 5) or Ii82-99 (lane 6) The experiment in Fig. 3B demonstrates that the WWCII sequence of DR3 impacts on binding to Ii.

The impact of TyrTrpAsp DRβ mutation on the assembly and transport of DR heterodimers

We examined, whether the TyrTrpAsp-mutated DR heterodimer is intracellularly transported in transfected cells. To monitor intracellular transport, the Asn-linked carbohydrate on DRβ chain, which is modified upon transport to Golgi compartments and there acquires endonuclease (Endo) H resistance was examined. In Fig. 4A immunoprecipitates from DR (upper panel) and from TyrTrpAsp-mutated DR (lower panel) transfected cells were treated with Endo H (lane 2), with peptide N-glycosidase F (PNgase F) (lane 3), which completely cleaves the N-linked glycan chain, or left untreated (lane 1). Western blotting of the DRβ chain revealed that both,

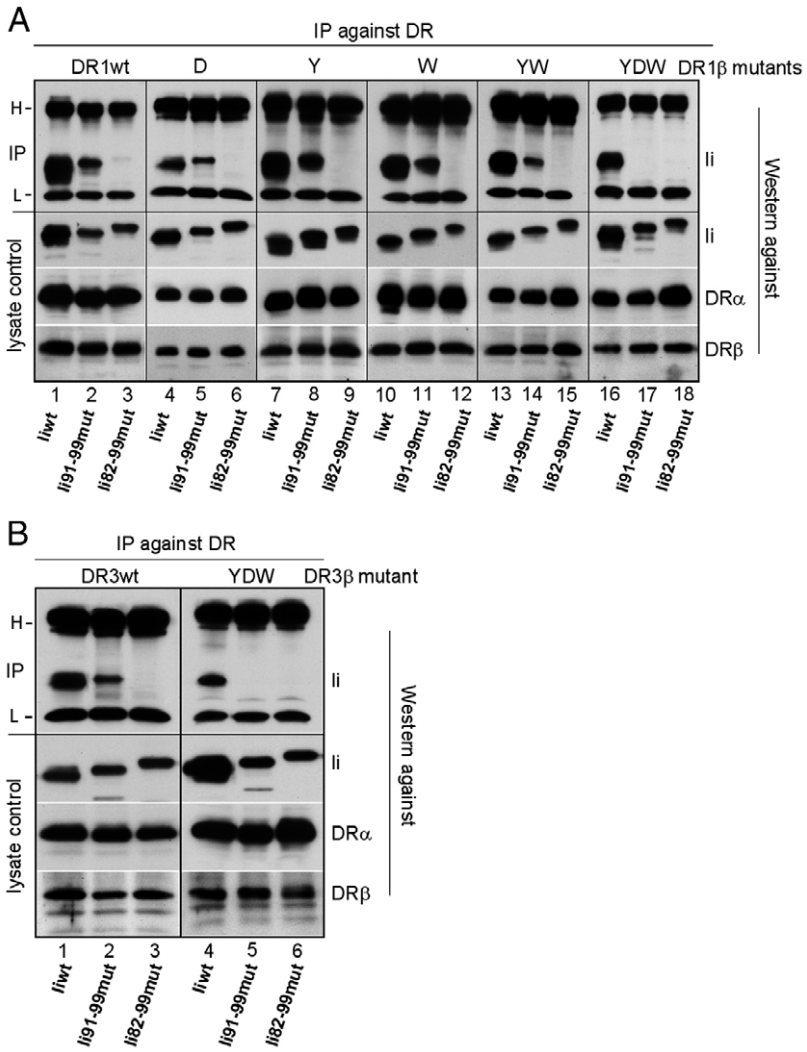

Fig. 3. Binding of Ii sequences to YWD DR1 mutants. (A) DR1β chain mutants (lanes 4 to 18) and DR1β chain (lanes 1 to 3) were co-expressed with DRα and tested for binding to full-length Ii (wt Ii, lanes 1, 4, 7, 10, 13, and 16), and to the Ii91-99 mutant with the MHCII groove-binding sequence aa 91-99 replaced by an irrelevant sequence (lanes 2, 5, 8, 11, 14, and 17), or to the mutant Ii82-99 where the complete MHCII-binding sequence including the proline-rich sequence of Ii was replaced by an unrelated sequence (lanes 3, 6, 9, 12, 15 and 18). Cell lysates were immunoprecipitated (IP) with I251 against DR. The immunocomplexes and cell lysates (lysate control) were separated by SDS-PAGE, immunoblotted and detected with antibodies against Ii or the DR subunits. Heavy (H) and light (L) chains are indicated. (B) The DR3β chain mutant (YWD to AAA) or the wild-type DR3β chain were co-expressed with DRα and full-length Ii (wt Ii, lanes 1 and 4), Ii91-99mut (lanes 2 and 5), or Ii82-99 mut (lanes 3 and 6). COS-7 cells were lysed in 0.5% NP40 and immunoprecipitated (IP) against DR (mAb I251). Subsequently, the immunoprecipitates were blotted against Ii (upper panel) and the lysates were immunoblotted with antibodies against Ii, DRα, or DRβ chains (lower panel).

the wild-type DRβ (lane 2, upper panel) and the TyrTrpAsp-mutated DRβ chain (lane 2, lower panel) show partial resistance to Endo H digestion. For comparison, untreated and PNgase-F-treated lysates (lanes 1, 3, 4 and 6) indicate the mobility of glycosylated and of non-glycosylated β chain bands. The band intensities within lane 2 show that about half of the amount of DRβ was transported to Golgi compartments, where the carbohydrates were modified (lane 2). In lanes 4 to

Fig. 4. Transport and assembly of YWD-mutated DRβ with the DRα chain. IMRS cells were transiently transfected with DRα and DRβ, or with DRα and YWD-mutated DRβ in the presence (lanes 4, 5, 6, 8 and 9) or absence (lanes 1, 2, 3 and 7) of Ii. Cells were lysed and DR was immunoprecipitated with mAb I251. Immunoprecipitates were digested with Endo H (E_H) (lanes 2 and 5), with PNgase F (P_F) (lanes 3 and 6) or left untreated (Ø) (lanes 1 and 4). Subsequently the samples were separated by SDS-PAGE and immunoblotted with S35 for DRβ. Lanes 7 and 8 show cell lysates western blotted for DRα. Ii was immunodetected in lane 9. (B) Endo H digestion of transfected DRβ chains. MelJuso cells were transfected with V5-tagged wtDRβ (lanes 1-3), or the mutants DRβY (lanes 4-6), DRβW (lanes 7-9), DRβD (lanes 10-12), DRβYW (lanes 13-15) and DRβYWD (lanes 16-17). Lysates were digested with EndoH or PNGaseF and analyzed by western blotting for the presence of the V5-tagged DRβ chains. (C) Cellular distribution of DRβ chains. MelJuso cells were transfected with V5-tagged DRβYWD, with wtDRβ or with empty expression vector (mock). Expression was monitored by double immunofluorescence staining of the cells with mAb V5 or DR mAb I251 (red staining, top row) and with an antibody against Cath B (green staining, middle row). In the bottom row, staining of rows 1 and 2 were merged. Nuclei were counterstained with DAPI. (D) MelJuso cells expressing V5-tagged wtDRβ or YWD-mutated DRβ were surface biotinylated and lysed with NP40. Cell lysates with wtDRβ or YWD-mutated DRβ were separated in lanes 3 and 4 or subjected to immunoprecipitation with V5 mAb. DRβ chains were immunoblotted with streptavidin peroxidase (lanes 1 and 2) or with V5 mAb. (E) Co-immunprecipitation of DRβ with Ii. MelJuso cells were transfected with V5-tagged wtDRβ or V5-tagged mutant DRβYWD. Ii was immunoprecipitated with polyclonal antibody S22 and the immunocomplexes were detected by western blotting with mAb V5 for the presence of wtDRβ (lane 1) or DRβYWD (lane 3). Expression of the V5-tagged β chains was examined by western detection of the lysates (lanes 2 and 4). (F) Western blotting of DRα immunoisolated with YWD mutant DRβ chain expressed in the presence or absence of wtDRβ. IMRS cells were transfected with DRα, DRβYWD, Ii and wtDRβ (lane 2) or with a vector control (lane 1). DRβYWD was immunoprecipitated and the immunocomplexes were western blotted for the presence of DRα. Expression of the individual molecules was examined by western blotting of the lysates with mAb 1B5 (DRα; lanes 4 and 5), mAb V5 (DRβYWD; lanes 6 and 7) and mAb 6D4 (wtDRβ; lane 8).

6, in addition to α and β chains, Ii was co-expressed. In the presence of Ii, wild-type DRβ chain acquires almost complete resistance to Endo H digestion indicating that Ii is a chaperone for MHCII molecules (lane 5, upper panel). Expression of Ii however, did not increase the amount of Endo-H-resistant TyrTrpAsp-mutated DRβ chain (lane 5, lower panel). This result indicates that expression of Ii did not enhance transport of the TyrTrpAsp-mutated DR molecules as shown for wild-type DR. Lanes 7 and 8 show expression of the α chain and lane 9 of Ii chain in cell lysates. We conclude that contact of Ii to WWCII is required to facilitate intracellular transport of DR heterodimers. Interaction of the proline-rich region of Ii to WWCII on the DRβ chain could be important to accomplish assembly of MHCII subunits.

To examine whether the WWCII domain impacts on DR assembly, we transfected MelJuso cells with mutant DRβ or with wild-type DRβ chains encoding cDNAs. MelJuso cells express HLA-DR and Ii polypeptides endogenously. Therefore, the transiently expressed DRβ chains will compete with endogenous DRβ chains for binding to DRα. Transfected MelJuso cells were lysed and lysates were subjected to Endo H (Fig. 4B lanes 2, 5, 8, 11, 14 and 17) or to PNgase F (lanes 3, 6, 9, 12, 15 and 18) treatment, or left untreated (lanes 1, 4, 7, 10, 13 and 16). The lysates were separated by SDS-PAGE and immunoblotted for the transiently expressed DRβ chains. The V5-tagged wild-type DRβ chain shows that a substantial proportion of this polypeptide contains Endo-H-resistant carbohydrates (lane 2). The glycan chains are completely

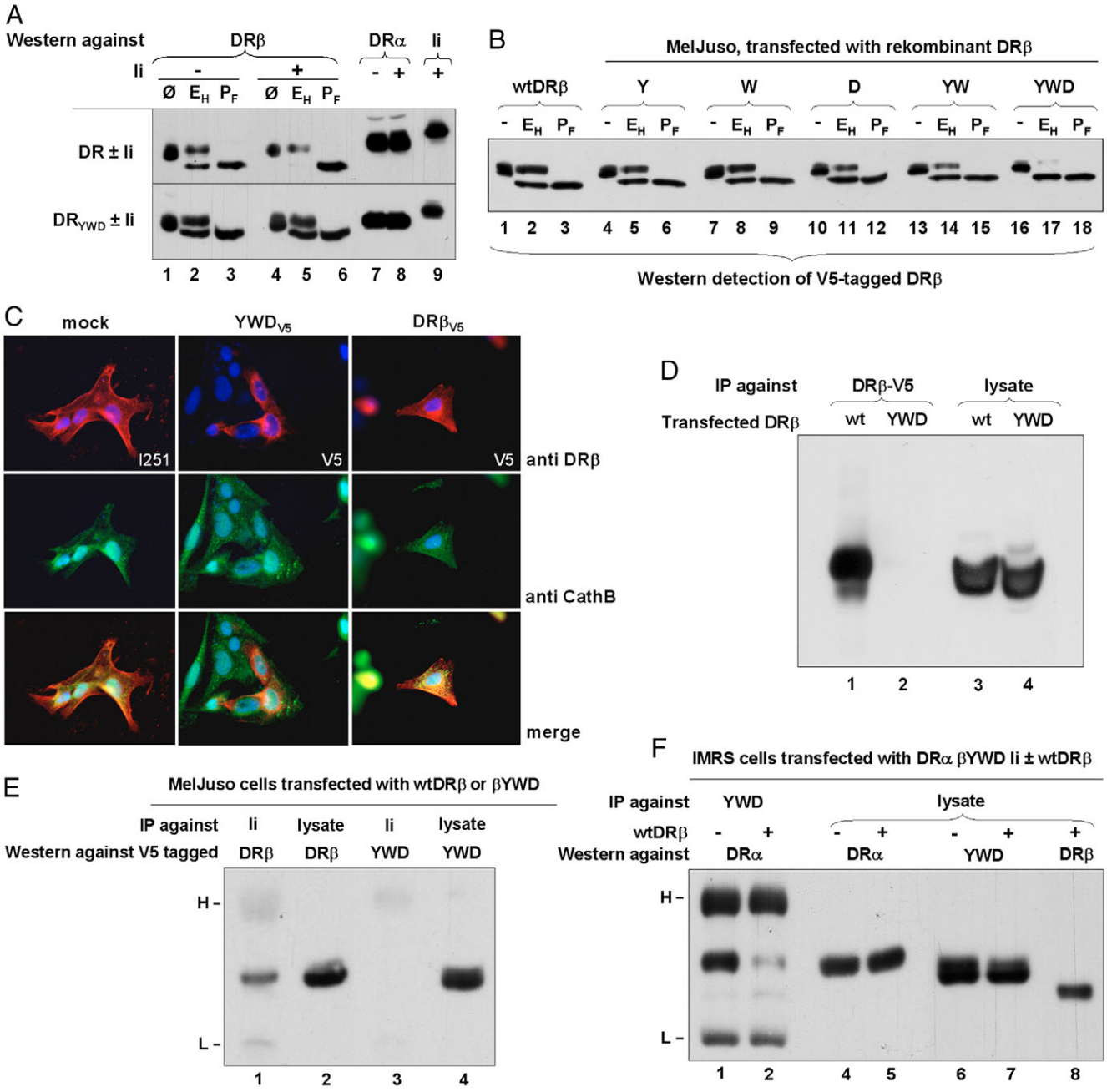

Fig. 4. See previous page for legend.

cleaved by PNgase F digestion (lane 3). Only assembled αβ heterodimers are exported from the ER to Golgi compartments, where N-linked carbohydrates are processed. Therefore, this result indicates that the V5-tagged wild-type DRβ chain can compete with the endogenous DRβ chain for binding to DRα. Similar results were obtained with Tyr, Trp, Asp, and TyrTrp DRβ mutants (lanes 4 to 15). These mutant DR chains are capable to assemble in the presence of endogenous DRβ to the DRα chain. By contrast, the TyrTrpAsp mutant DRβ chain, which has a deficiency for interaction to the proline-rich sequence of Ii appears in transfected MelJuso cells almost completely Endo H sensitive (lane 17). This result indicates that TyrTrpAsp-mutated DRβ is not transported in MelJuso cells and possibly retained in the ER. We inspected localization of the V5-tagged TyrTrpAsp-mutated DRβ and of the V5-tagged wtDRβ chains in endosomal and lysosomal compartments by co-staining of MHCII with the endosomal and lysosomal marker cathepsin B (Cath B). Mock-transfected MelJuso cells (left, upper panel) show bright intracellular staining and strong surface expression detected with the DR-specific mAb I251. Co-staining with an antibody against Cath B (left, middle line) and merging (left, lower line) indicates the presence of a proportion of MHCII molecules in Cath-B-containing compartments. A similar result was obtained by co-staining of V5-tagged wtDRβ chain with Cath B (right panel). The TyrTrpAsp DRβ mutant expressed in MelJuso cells (middle panel) however exhibits a perinuclear staining of MHCII and almost no merging with co-stained Cath B (middle, lower line). Moreover, co-staining with calnexin revealed that the TyrTrpAsp-mutated DRβ chain is largely contained in ER vesicles, when expressed in MelJuso cells (data not shown).

To verify, that the TyrTrpAsp-mutated DRβ chain is not expressed on the cell surface, MelJuso cells expressing V5-tagged wtDRβ or TyrTrpAsp-mutated DRβ chains were surface biotinylated (Fig. 4D). Cell lysates separated in lanes 3 and 4 were blotted for V5-tagged DRβ chains. This control indicates that similar amounts of V5-tagged wild-type and TyrTrpAsp-mutated DRβ chains were expressed by the transfected MelJuso cells. Immunoprecipitation of the V5-tagged DRβ chains (lanes 1 and 2) and subsequent detection of biotinylated DR showed that only wtDR was detected (lane1). The TyrTrpAsp-mutated DRβ chain was not isolated from cell-surface-labeled MeJuso cells (lane 2).

We investigated whether the transiently expressed DRβ chains are contained in complexes with Ii. Ii was immunoprecipitated from MelJuso cells, which transiently express V5-tagged wild-type or TyrTrpAsp mutant DRβ chains. Fig. 4E, lanes 1 and 3 show Ii immunoprecipitates and lanes 2 and 4 cell lysates, which were immunoblotted for the transiently expressed DRβ chains. V5-tagged DRβ chain co-isolates with Ii (lane 1), whereas the TyrTrpAsp DRβ mutant was not detected in Ii immunoprecipitates (lane 3). Immunoblotting of cell lysates revealed that similar amounts of both V5-tagged DRβ chains were expressed (lanes 2 and 4). This result suggests that in MelJuso cells, the TyrTrpAsp-mutated DRβ chain is not contained in a complex with Ii and possibly not with DRα. To examine whether interaction of TyrTrpAsp-mutated DRβ with DRα is influenced by expression of wild-type DRβ, we used human IMRS cells, which do not express endogenous MHCII or Ii. IMRS cells were transfected with DRα, TyrTrpAsp-mutated DRβ, Ii and with or without cDNAs encoding wild-type DRβ (Fig. 4F). The V5-tagged TyrTrpAsp mutant was immunoprecipitated and immunoblotted with mAb for DRα. In the absence of wild-type DRβ, the TyrTrpAsp mutant co-isolates DRα (lane 1). Co-expression of wild-type DRβ (lane 2) strongly reduces the co-isolation of DRα with the TyrTrpAsp mutant. Expression of the transiently expressed polypeptides is shown in lanes 4 to 8. This result indicates that the transfected wild-type DRβ chain almost completely inhibits binding of the TyrTrpAsp mutant DRβ chain to DRα.

Discussion

A novel domain on the DRβ chain regulates the chaperone role of invariant chain

The invariant chain facilitates assembly and subsequent intracellular transport of DR molecules. In the absence of Ii, DR subunits form aggregates that are retained in the ER and only a proportion of DRαβ heterodimers acquires a transport-competent conformation and is exposed on the cell surface (Marks et al., 1995). Interaction of Ii with the MHCI groove appears to chaperone assembly of MHCII subunits (Romagnoli and Germain, 1994). A peptide, covalently bound to the N-terminus of β chain was shown to stabilize class II αβ dimers (Kozono et al., 1994). These tethered αβ-peptide complexes are detected on the cell surface of transfected B cells or fibroblast cells. Surface expression of functional class II peptide receptors could suggest that binding of a peptide to the class II groove is sufficient to chaperone assembly of αβ dimers. However, further studies indicated that this αβ-peptide complex has a binding site for Ii other than the peptide-binding groove, suggesting a role of Ii for surface expression of the tethered αβ-peptide complex (Wilson et al., 1998). This observation is consistent with our finding that interaction of Ii with MHCII is mediated by conserved residues in the β2 domain of the β chain.

In this report we describe a novel domain (WWCII) on the DRβ chain, which provides an additional binding site for Ii. We suggest that interaction of Ii with WWCII on DRβ chain facilitates folding and subsequent intracellular transport of DR heterodimers. Alteration of this domain by site-directed mutagenesis results in αβ dimers, which are isolated by a mAb that reacts to the heterodimer but not to single DR chains. In addition, the mutated αβ dimers bind to an antigenic sequence contained in an Ii fusion protein, although the TyrTrpAsp mutant DRβ chain binds with reduced affinity. Moreover, it was shown that in transfected IMRS cells, the TyrTrpAsp-mutated DR heterodimer is transported, which was demonstrated by Endo H resistance of the β chain. Hence, despite the mutations, important molecular functions of class II are maintained. Our results indicate that interaction of the proline-rich sequence of Ii to DR dimers is abolished by mutation of TyrTrpAsp residues on the DRβ chain. This was concluded, because a groove-binding-sequence-deleted Ii chain did not bind to the TyrTrpAsp-mutated DR1 and to the DR3 allotypes. The importance of WWCII for class II assembly was demonstrated by transfection of MelJuso cells, which express endogenous DR chains, with the TyrTrpAsp-mutated DRβ cDNA. In these cells, endogenous DRβ chain out-competes binding of TyrTrpAsp-mutated DRβ to DRα. In contrast to the other mutants in MelJuso cells, the TyrTrpAsp-

mutated DRβ chain is not transported and is not contained in complexes with Ii. This latter finding is consistent with the observation that Ii does not co-isolate with single DRβ chain (Neumann and Koch, 2005). The role of Ii for assembly of DRα with wtDRβ was demonstrated by transfection of IMRS cells, which are negative for Ii and DR. Only in the presence of Ii, was the wtDRβ chain capable of competing with the TyrTrpAsp-mutated DRβ chain for binding to DRα. Hence, we suggest that interaction of Ii with WWCII on the DRβ chain governs the chaperon function of Ii.

A binding site for invariant chain is located on the β2 domain of DRβ chain

Our data suggest that the WWCII architecture on MHCII molecules provides a platform for the binding of residues 82-87 of Ii. We discovered the WWCII sequence by comparison of MHC class II β chain sequences to a WW motif found in a family of proteins. Comparison of the WW domain of dystrophin as a member of the WW protein family (Zarrinpar and Lim, 2000; Huang et al., 2000) with MHCIIβ chain sequences revealed in both sequences two tryptophans at a distance of 22 aa residues. The WWCII sequence contains a pair of conserved W residues at position 131 and 153 of the DRβ chain. The structure of WWCII however differs from the WW domain. WWCII, with a size of at least 50 residues, is larger than the WW motif with 38 to 40 residues. A WW domain, as found in dystrophin, adopts an anti-parallel three-stranded fold, where the second Trp residue forms a hydrophobic pocket with a Tyr residue. The groove formed by the conserved Tyr and Trp residues is composed of nearly parallel aromatic residues where a proline-rich motif could bind. In contrast to the WW protein family, in WWCII the pair of Trp residues is flanked by sequences containing Tyr123 and Tyr171 residues. The upper pocket consisting of Trp153 and Tyr123 residues is directed towards the surface, whereas the hydrophobic pocket formed by Tyr171 and Trp131 is completely turned to the inside of the molecule. The membrane proximal Trp131 and Tyr171 pair is part of the Ig-domain structure and a similar structure is found in the protein sequence of other members of the Ig super gene family. The pair of Trp153 and Tyr123 residues however, is contained only in the sequence of the MHCIIβ chains.

The WWCII structure on MHCII β chains may define a novel protein motif that interacts with proline-containing sequences. For comparison, WW domains contain Trp and Tyr residues, which form binding pockets for proline residues. WW domains have highly diverse sequence preferences and binding of Pro residues depends on adjacent residues. The proline-rich sequence motif of human Ii matches the sequence of the group III WW motif, where polyproline is flanked by Lys or Arg residues (Bedford et al., 2000; Sudol and Hunter, 2000). In WWCII, the Trp153 and Tyr123 residues partially define the binding sequence to the proline-rich sequence of Ii. For the proline-rich motif ProLysProProLysPro corresponding to residues 82-87 of human Ii, it was shown that mutation of the flanking Pro and of Lys, to Leu residues strongly reduced binding of this sequence to DR molecules (Sievers et al., 2002). The Lys residues are conserved in the proline-rich Ii sequence of six mammalian species. It is possible that a positively charged Lys residue of Ii interacts to a negatively charged residue on the DRβ chain. The tertiary structure of HLA-DRβ chains revealed Trp153 and adjacent Tyr123 as conserved residues. In addition to the TrpTyr pocket, the ϵ carboxyl group of Asp152 is accessible for inter-chain contacts. Thus, interaction of Lys83 of Ii, with Asp152 of the DRβ chain might be possible. We therefore suggest that interaction of WWCII to Ii depends on binding of TrpTyr and Asp residues on DRβ chain to Pro and Lys residues on Ii.

We found a novel domain on DRβ chains that mediates contact with a proline-rich sequence of Ii. The conserved WWCII sequence may play a central role for assembly of α, β and Ii subunits. In antigen-presenting cells, where the numerous class II subunits of various allo- and isotypes are expressed, association of matched α and β heterodimers could be monitored by interaction of Ii with WWCII on the β chain.

Materials and Methods

PCR-based mutagenesis

The coding sequence for HLA-DR1*010101, HLA-DR3*030101 beta chains and of human Ii were subcloned from pCEXV3 into expression vector pcDNA3.1(–) and were used as template for PCR mutagenesis. The residues Tyr123, Trp153 and Asp152 of DRβ were mutated to Ala. Mutation of all three residues within the same chain was obtained by introducing the mutation for Asp152Ala and Trp153Ala with one pair of mutagenic primers, while the third mutation Tyr123Ala was introduced sequentially. For each DR1 construct, two fragments encompassing the mutation were PCR-amplified in reactions containing either DR1uni (5′-CACCATGGTACCGTGCACGCTGCTC-3′) and the mutagenic anti-sense primers or DR1rev (5′-TCAGCTCAGGAATCCTGTTGG-3′) and the mutagenic sense primers. The mutagenic sense and anti-sense primers are as follows: W131Auni (5′-CTGGCCGTTCCGGAACGCCCTGACTTCAATGC-3′); W131Arev (5′-GCATTGAAGTCAGGCGTTCCGGAACGGCCAG-3′); D152Auni (5′-CTGATCCAGAATGGAGCTTGGACCTTCCAG-3′); D152Arev (5′-CTGGAAGGTCCAAGCTCCATTCTGGATCAG-3′); Y123Auni (5′-GTGAGTGGTTTCGCTCCAGGCAGC-3′); Y123Arev (5′-GCTGCCTGGAGCGAAACCACTCAC-3′), D152A.W153Auni (5′-CAGAATGGAGCTGCGACCTTCCAG-3′); D152A.W153rev (5′-CTGGAAGGTCGCAGCTCCATTCTG-3′). For DR3: DR3uni (5′-CACCATGGTGTGTCTGAGGCTCCCTG-3′); DR3rev (5′-TCAGCTCAGGAATCCTCTTGGCTG-3′). The mutagenic primers D152A.W153Auni (5′-CACAATGGAGCCGCGACCTTCCAGACCCTGGTG-3′); D152A.W153Arev (5′-GGTCTGGAAGGTCGCGGCTCCATTGTGGATCAG-3′); Y123Auni (5′-GTGAGTGGTTTCGCTCCAGGCAGCATTG-3′) and Y123Arev (5′-CAATGCTGCCTGGAGCGAAACCACTCAC-3′). For DR4: DR4uni (5′-CACCATGGTGTGTCTGAAGTTCCCTG-3′); DR4rev (5′-TCAGACCGTGCTCCTCCATTCCAC-3′). The mutagenic primers D152A.W153Auni (5′-CTGATCCAGAATGGAGCCGCGACCTTCCAGACC-3′); D152A. W153Arev (5′-GGTCTGGAAGGTCGCGGCTCCATTCTGGATCAG-3′); Y123Auni (5′-CACAATGGTTTCGCTCCAGGCAGCATT-3′); and Y123Arev (5′-CAATGCTGCCTGGAGCGAAACCATTCAC-3′).

Constructs of human Ii were prepared, where CLIP(91-99) was either deleted or substituted by a non-binding sequence, or where CLIP(82-99) was completely replaced by a non-MHC binding sequence. The mutations were introduced with the primers Ii33uni (5′-CCAGTCATGGATGACCAGCGC-3′), Ii33rev (5′-CAGATGGAGATTGGGCAGCAGG-3′) and the mutagenic primers Ii33Δ91-99uni (5′-CAGGCGCTGCCCATGGGAGCC-3′); Ii33Δ91-99rev (5′-GGCTCCCATGGGCAGCGCCTGCTTGCTCACAGGCTTGGGAGG-3′); Ii33.91-99uni (5′-CAGGGTGATGTAAATGAAATCCAAAAGCAGGCGCTGCCCATGGGAGC-3′); Ii33.91-99rev (5′-CTTTTGGATTTCATTTACATCACCCTGCTTGCTCACAGGCTTGGGAGG-3′); Ii33.82-99uni (5′-AGTGATATTAATTGCACCAAAGTTTTACAGGGTGATGTAAATGAAATC-3′); and Ii33.82-99rev (5′-TAAAACTTTGGTGCAATTAATATCACTAAGCTTCATGCGCAGGTTCTC-3′). In a second reaction, the fragments were annealed, extended and amplified with DR1uni and DR1rev for the DR1 constructs, for DR3 with DR3uni and DR3rev, for DR4 with DR4uni and DR4rev and for Ii33 with Ii33uni and Ii33rev, and were cloned into pcDNA3.1(–)neo.

Cells and antibodies

MelJuso cells were provided by Dr G. Moldenhauer [German Cancer Research Center (DKFZ), Heidelberg, Germany]. IMRS cells were purchased from the Coriell Institute for Medical Research and COS-7 cells were obtained from the American Type Culture Collection. The rabbit antisera S22 and S35 (Koch et al., 1987), the mouse monoclonal antibodies anti-DR I251 (Pesando and Graf, 1986), anti-Ii33 Bu43 (Wraight et al., 1990), anti-DRα 1B5 (Adams et al., 1983) and 6D4 (Neumann, 2005) have been described. Monoclonal antibody V5 was purchased from Invitrogen. Rabbit serum to human cathepsin B was purchased from Molecular Probes (Leiden, The Netherlands) and anti-calnexin mAb clone 37 was purchased from BD (Heidelberg, Germany).

Immunofluorescence microscopy

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were blocked with 10% BSA and stained with the primary antibody, followed by Alexa Fluor-labeled secondary antibodies [goat anti-mouse IgG (594 nm) or goat anti-rabbit IgG (488 nm)]. The cells were mounted with Mowiol and visualized by fluorescence microscopy (Axiophot, Zeiss).

Transfection

MelJuso cells and IMRS cells were transfected with the transfection reagent JetPei (QbioGene). The cells were transfected in a six-well culture plate at 50% confluence. The DNA-polyethylenimine complexes were prepared as recommended by the supplier. COS-7 cells were transfected with the liposomal transfection reagent DOSPER (Roche). DNA mixed with DOSPER was incubated for 20 minutes and added to cells. After 48 hours, transfected cells were harvested and subjected to immunoprecipitation, metabolic radiolabeling or immunofluorescence microscopy.

Metabolic radiolabeling and immunoprecipitation

Cells were incubated in methionine-free medium for 45 minutes and labelled for 15 minutes with 50 μCi [^{35}S]methionine as recently described (Neumann et al., 2001). Cells were lysed in 0.5% NP40 (Sigma) in 20 mM Tris-HCl, pH 7.4 and precleared with CL4B-Sepharose (Amersham Pharmacia); immunoprecipitation was performed with protein-A-Sepharose. Samples were analyzed by reducing SDS-12% PAGE followed by autoradiography or western blotting of unlabeled cells. Transfected MelJuso cells were biotinylated with Sulfo-Biotin-7-NHS according to the instruction of the supplier (Pierce, Bonn, Germany). In brief, 1×10^7 cells were suspended in 1 ml biotinylation buffer (50 mM boric acid and 150 mM NaCl). 10 μl sulfosuccinimidyl-7-biotinamido-6-hexanamidohexanoate (10 mg/ml in H_2O) were added and incubated for 15 minutes. The reaction was stopped by addition of 20 μl of 100 mM NH_4Cl. The samples were washed twice in ice-cold PBS and stored at –70°C.

Immunoblotting

Cell lysates were prepared with 0.5% NP40 in PBS with 10 mM Tris-HCl, pH 7.4 containing Complete (Roche) protease inhibitor mix. The samples were separated by reducing SDS-12% PAGE and electrophoretically transferred onto an Immobilon P membrane (Millipore). The membrane was blocked for 1 hour in PBS-Roti-Block (Roth), washed with PBS-0.2% Tween 20 and probed with the primary antibody followed by the peroxidase-conjugated secondary antibody and ECL chemiluminescent substrate (Pharmacia).

This work was supported by Grant Ko 810/6-3 from the Deutsche Forschungsgemeinschaft. We thank Angelika König for technical assistance.

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