Expression of Recombinant HLA-DR2 Molecules

REPLACEMENT OF THE HYDROPHOBIC TRANSMEMBRANE REGION BY A LEUCINE ZIPPER DIMERIZATION MOTIF ALLOWS THE ASSEMBLY AND SECRETION OF SOLUBLE DR αβ HETERO DIMERS*

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Major histocompatibility complex (MHC) class II molecules are membrane-anchored heterodimers that present peptides on the surface of antigen presenting cells to T cells. Soluble HLA-DR2 molecules were expressed for structural and functional characterization of the MHC/peptide/T cell receptor recognition unit. The α and β chains of DR2 (encoded by the DRA, DRB1-1501 genes) did not assemble in mammalian or insect cell lines when the transmembrane regions of one or both chains were truncated. The hydrophobic transmembrane regions of DRα and DRβ facilitate assembly of the heterodimer and were therefore replaced by the leucine zipper dimerization motifs from the transcription factors Fos and Jun, which assemble as a soluble, tightly packed coiled coil structure. The DRα-Fos and DRβ-Jun constructs were expressed in a methylotrophic yeast, Pichia pastoris, using the mating factor secretion signal to direct expression to the secretory pathway. DR αβ heterodimers were purified from supernatants using an antibody specific for the DR αβ heterodimer. Kinetic and quantitative peptide binding experiments demonstrated that recombinant DR2 molecules were efficiently loaded with an antigenic peptide. Soluble DR2 molecules can be used to define structural aspects of the MHC/peptide/T cell receptor interaction and to study the signals induced by T cell receptor recognition of soluble DR2 peptide complexes.

MHC class II molecules determine the specificity of T cell-mediated immune responses by binding peptides from foreign antigens in an intracellular processing compartment and by presenting these peptides on the surface of antigen presenting cells to T cells (for review, see Strominger and Wiley (1995)). MHC genes are highly polymorphic; with 137 known alleles, the DRβ chain gene (DRB1) is the most polymorphic human gene that has been identified (Marsh and Bodmer, 1995). The polymorphic residues are clustered in the peptide binding site and thereby define the repertoire of peptides that are presented to T cells (Bjorkman et al., 1987; Stern et al., 1994). Some alleles of MHC class II genes confer susceptibility to autoimmune diseases, probably through the presentation of pathogenic self-peptides. For example, HLA-DR2 confers an increased risk for multiple sclerosis, while subtypes of HLA-DR4 confer susceptibility to rheumatoid arthritis (for reviews, see Todd et al. (1988) and Wucherpfennig and Strominger (1995b)).

Soluble, empty MHC class II molecules are required for crystallographic studies of single MHC-peptide complexes and for studying the biochemical interaction of MHC-peptide complexes with the T cell receptor. Structural characterization of the MHC-peptide/T cell receptor recognition unit will provide important insights into the mechanisms by which MHC molecules confer susceptibility to autoimmunity. Soluble MHC-peptide complexes may also be useful for the treatment of autoimmune diseases. Studies in the experimental autoimmune encephalomyelitis model have demonstrated that an autoimmune disease can be treated by the administration of soluble MHC-peptide complexes (Sharma et al., 1991).

MHC class II molecules can be purified by affinity chromatography following detergent solubilization of membranes (Gorga et al., 1987); however, MHC molecules purified from B cell lines have passed through the MHC class II peptide loading compartment and are therefore already loaded with a diverse set of peptides (Chicz et al., 1992). Soluble HLA-DR1 and HLA-DR4 molecules were expressed in insect cells using cDNA constructs of the DRα and DRβ extracellular domains (Stern and Wiley, 1992). Expression of other MHC class II molecules has been difficult due to a failure of MHC class II α and β chains to assemble and/or due to a strong tendency for molecules to aggregate, even in the presence of a peptide ligand. These results may be explained by the observation that the transmembrane regions of the MHC class II α and β chains facilitate the proper assembly of the αβ heterodimer, presumably through the interaction of the two α-helical transmembrane segments (Cosson and Bonifacio, 1992). Transmembrane interactions are also important for the assembly of other multipeptide complexes, such as the T cell receptor complex (Manolios et al., 1990).

The transmembrane regions were important for the assembly of DR2 molecules since the α and β chains did not assemble in mammalian or insect cells when the transmembrane regions were truncated. The leucine zipper dimerization motifs from the transcription factors Fos and Jun were therefore used to replace the hydrophobic transmembrane regions. Synthetic peptides of the Fos and Jun leucine zipper dimerization motif are known to assemble as stable, soluble heterodimers (O’Shea et al., 1989). The leucine zippers are characterized by five leucines that are spaced periodically at every seventh residue

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The abbreviations used are: MHC, major histocompatibility complex; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; MBP, myelin basic protein.
GS115 cells were grown to mid-log phase in YPD media (1% yeast GS115 strain (following the procedure provided by Invitrogen). Briefly, AOX interface between the two chain constructs was examined by PCR analysis of genomic DNA isos-AAA AGA GAG GGG GAC ACC CGA CCA CGT TTC 3 Eco RI site underlined (encodes 3 gene). Transformants that had integrated the plasmid DNA into the AOX1 locus showed little or no growth on methanol plates due to disruption of the alcohol oxidase gene.

Identification of Recombinant Colonies—Integration of DRa and DRb chain constructs was examined by PCR analysis of genomic DNA isolated from individual colonies. Recipient colonies were transferred into 200 µl lysis buffer (25 mM LiCl, 50 mM Tris, pH 8.0, 4% Triton X-100, 62 mM EDTA) using a sterile toothpick. Acid-washed glass beads and an equal volume of phenol/chloroform (1:1) were added, and samples were vigorously vortexed. Following centrifugation, the upper phase was transferred to a clean tube, and genomic DNA was precipitated by addition of 2.5 volumes of cold EtOH. Following incubation at -20°C for 20 min, the pellet was collected by centrifugation, washed with cold 70% EtOH, and air-dried. DNA was resuspended in 40 µl of sterile water and denatured at 94°C for 10 min; 10 µl of DNA was used for each PCR reaction. DRa and DRb chains were amplified by PCR for 35 cycles (94°C 1 min, 55°C 2 min, 72°C 2 min) using the oligonucleo-}

**MATERIALS AND METHODS**

DNA Constructs—The extracellular domains of DRa and DRb were expressed as fusions with the leucine zipper dimerization motifs of Fos or Jun. The DRa and DRb gene products were fused to the DRa and DRb C-termini in pPIC9 plasmid DNA was purified on CsCl gradients and digested with

**Expression and Purification of HLA-DR2 Heterodimers—**

Assembly of HLA-DR2 by a Leucine Zipper Dimerization Motif

**RESULTS**

Replacement of the Transmembrane Region with the Leucine Zipper Dimerization Motif Allows Assembly and Secretion of HLA-DR2 Heterodimers—Soluble HLA-DR1 and HLA-DR4 have been expressed in insect cells using cDNA constructs for the extracellular domains of DRa and DRb (Stern and Willey, 1992). When this strategy was attempted for the expression of HLA-DR2 (using the DRA, DRB1-1501 genes), DR a heterodimers could not be detected in cell lysates or supernatants of infected Sf9 cells, even though separate DRa and b chains could be readily detected in cell lysates by Western blot analysis. The requirements for the assembly of DR2 heterodimers
were therefore examined by transfecting mammalian cells (Chinese hamster ovary cells) with different cDNA constructs. The DRα and DRβ transmembrane regions could be replaced with a glycan-phosphatidylinositol anchor from human placental alkaline phosphatase (Wettstein et al., 1991); however, DR2 molecules were not assembled when the transmembrane region of one chain was truncated (data not shown). These results strongly suggested that interaction of the transmembrane regions of DRα and DRβ was important for the assembly of DR2.

The transmembrane regions of DRα and DRβ were replaced with the leucine zipper dimerization motifs from the transcription factors Fos and Jun, which form stable heterodimers in solution (Figs. 1 and 2). The extracellular domains of DRα (residues 1–191) and DRβ (residues 1–198) were fused in frame with a 7-amino acid linker (VGDGGGG that contained a SalI restriction site) and the 40-amino acid leucine zipper domains of Fos (DRα-Fos) or Jun (DRβ-Jun) (van Straten et al., 1983; Angel et al., 1988). The extracellular domains ended with DRα 191 E) and DRβ 198 K) because charge-charge interactions between these two residues are thought to facilitate assembly (Cosson and Bonifacino, 1992). Since the leucine zipper has a tightly packed coiled coil structure that is stable in solution, cDNA constructs in which the transmembrane regions of DRα and DRβ were replaced with the Fos and Jun leucine zipper motifs were used to express HLA-DR2 in mammalian cells and in the yeast P. pastoris.

Expression of Soluble HLA-DR2 in P. pastoris, a Methylophilic Yeast—For protein production, the DRα-Fos and DRβ-Jun constructs were expressed in P. pastoris under the control of the alcohol oxidase (AOX1) promoter. P. pastoris was chosen because stable transformants can be rapidly generated and screened; in addition, several secreted proteins have been produced at very high levels in this system (Cregg et al., 1987). To direct expression to the secretory pathway, DRα and β chains were cloned into P. pastoris expression vector pPIC9 as in-frame fusions with the α-mating factor secretion signal (Fig. 3) (Brake, 1990). The α-mating factor secretion signal is cleaved by the KEX2 gene product (Leu-Glu-Lys-Arg ↓ Glu; cleavage C-terminal to Arg). Although this design results in the addition of a glutamic acid residue to the N terminus of the mature DRα and DRβ chains (see “Materials and Methods”), the N termini of these chains are located in a manner that this additional residue should not affect the assembly of the heterodimer. Molecules expressed as fusions with the α-mating factor secretion signal were efficiently secreted, while usage of the PHO1 secretion signal (vector pHIL-S1, Invitrogen) resulted in little or no secretion.

For transformation, the expression cassette of pPIC9 (Fig. 3) can be excised as a BglII fragment; the cassette carries 5′- and 3′-sequences of the AOX1 gene to allow for integration into the
AOX1 locus as well as the H1S4 gene that allows for selection of transformants in histidine deficient media. Genes integrate into the AOX1 locus by homologous recombination; integration into the AOX1 gene disrupts the gene and leads to slow growth if methanol is the only carbon source (methanol utilization deficient phenotype, Mut\(^a\)) (Cregg et al., 1987).

A major advantage of the P. pastoris system is that transformants can be readily identified. Integration into the AOX1 locus confers a methanol utilization deficient (Mut\(^a\)) phenotype that can be determined by comparing the growth of duplicate colonies on plates with methanol or dextrose as the sole carbon source. Mut\(^a\) colonies obtained after cotransformation of plasmids carrying the DR\(\alpha\) and DR\(\beta\) chain constructs (see "Materials and Methods") were tested by PCR analysis of genomic DNA for the integration of DR\(\alpha\) and \(\beta\) chain genes. 27 of 28 colonies with a Mut\(^a\) phenotype carried DR\(\alpha\) and/or DR\(\beta\) chain genes; four of these colonies (14.2%) had integrated both genes (Fig. 4).

Assembly and Secretion of Soluble HLA-DR2 Molecules—The four transformants that carried both DR\(\alpha\) and \(\beta\) chain genes were examined for the expression of DR2 heterodimers. Cells were grown for 2 days in media containing glycerol as the sole carbon source and were then switched to media containing 0.5% methanol. Supernatants and cell lysates were examined for the expression of DR2 heterodimers. Cells grown for 2 days in media containing 0.5% methanol. Colonies carrying only DR\(\alpha\) or DR\(\beta\) chain genes were used as controls. DR\(\alpha\), DR\(\beta\), DR\(\alpha\) and \(\beta\).

For large scale expression, cells were grown in a high density fermentor, and DR2 molecules were purified from concentrated supernatants by affinity chromatography. The mAb used for purification (L243) binds to the DR\(\alpha\) chain but only when properly assembled with the DR\(\beta\) chain. Affinity purification yielded approximately 300–400 \(\mu\)g of HLA-DR2/liter of culture. SDS-PAGE revealed two bands (Fig. 6A); the identity of these bands (upper band DR\(\alpha\), lower band DR\(\beta\)) and appropriate cleavage of the \(\alpha\)-mating factor signal peptide were confirmed by N-terminal sequence analysis following separation of DR\(\alpha\) and \(\beta\) chains by SDS-PAGE and transfer to a polyvinylidene difluoride membrane.

HPLC gel filtration analysis demonstrated that HLA-DR2 eluted as a single symmetric peak, demonstrating that the recombinant protein was not aggregated (Fig. 6B). HLA-DR1 expressed in the Baculovirus system was found to aggregate unless these molecules were loaded with a high affinity peptide (Stern and Wiley, 1992). These data demonstrated that the DR \(\alpha\) heterodimer was assembled and secreted in the P. pastoris expression system. Importantly, the purified molecules did not aggregate even though they had not been loaded with a high affinity peptide.

Loading of Soluble HLA-DR2 Molecules with a High Affinity Peptide—A human myelin basic protein (residues 85–99) that is recognized by DR2-restricted T cell clones from multiple sclerosis patients was previously shown to bind with high affinity (IC\(_{50}\) of 4.2 nM) to detergent soluble DR2 purified from L cell transfectants (Wucherpfennig et al., 1994, 1995a). A biotinylated peptide (biotin-SGSG-ENPVVHFFKNIYTPR with SGSG as a spacer between the biotin moiety and the MBP sequence) was used to examine the specificity of peptide binding to recombinant DR2. Peptide binding was assessed by in-
cubating DR2 molecules with the biotinylated peptide for different periods of time; nonbiotinylated peptide was used as a competitor to demonstrate the specificity of binding (Fig. 7). DR2-peptide complexes were then captured on an ELISA plate using the L243 mAb, and the amount of bound biotinylated peptide was quantitated using peroxidase-labeled streptavidin. Peptide binding to DR2 was strongly dependent on the pH, with a maximum observed at pH 7–8; relatively little binding was observed at pH 5. A similar pH optimum had previously been observed for binding of the MBP peptide to detergent-soluble DR2 (Wucherpfennig et al., 1994). Binding of peptide was dependent on the relative molar ratio of DR2 to peptide, with a maximum of binding at a 10-fold molar excess of peptide over DR2 (Fig. 7A). Binding was specific because it could be blocked by an excess of nonbiotinylated MBP(85–99) peptide but not by an analog peptide in which the P1 anchor residue of MBP(85–99) (residue 89, valine) was substituted by aspartic acid. DR2 (200 nM) was incubated for 24 h at 37°C with biotinylated peptide (2 μM) at pH 7.2 in the presence of competitor peptide (0–100 μM). DR2-bound biotinylated peptide was quantitated as described above.

To determine what fraction of recombinant molecules could be loaded with a single peptide, complexes of DR2 and the biotinylated MBP peptide were precipitated with streptavidin beads (Fig. 8). Following precipitation, DRα and β chains were resolved by SDS-PAGE and detected by Western blotting using a polyclonal DR antiserum. Approximately 50% of the molecules were precipitated with streptavidin beads (Fig. 8, lane 3),
Assembly of HLA-DR2 by a Leucine Zipper Dimerization Motif

Kinetics of Peptide Binding to Soluble HLA-DR2 Molecules—

The kinetics of peptide binding by detergent-soluble DR2 purified from an Epstein-Barr virus-transformed B cell line and by recombinant DR2 were compared (Fig. 9). The kinetics of peptide binding were strikingly different. With recombinant molecules, the kinetics of binding were much faster, and a much larger fraction of recombinant molecules could be loaded with a biotinylated peptide. These results demonstrate that recombinant molecules can be efficiently loaded with a single peptide, while the peptide binding site of DR molecules from B cells is already occupied with high affinity peptides.

50% remained in the supernatant (lane 4). Control experiments demonstrated that precipitation of the DR2-peptide complexes was specific as the molecules were not precipitated when control agarose beads (lane 5), an unlabeled MBP peptide (lane 7), or an excess of unlabeled peptide over biotinylated peptide were used (lane 8); rather the DR2 molecules remained in the unbound fraction (lanes 6, 8, and 10).

KineticsofPeptideBindingtoSolubleHLA-DR2Molecules—

The kinetics of peptide binding by detergent-soluble DR2 purified from an Epstein-Barr virus-transformed B cell line and by recombinant DR2 were compared (Fig. 9). The kinetics of peptide binding were strikingly different. With recombinant molecules, the kinetics of binding were much faster, and a much larger fraction of the molecules were loaded (50% maximum binding after only 3 h). In contrast, the kinetics of peptide binding to DR2 from B cells were slow; the fraction of peptide loaded molecules slowly increased over a 48-h period without reaching a plateau (Fig. 9). These results are explained by the fact that the majority of DR molecules purified from B cells are already occupied with high affinity peptides, as demonstrated by peptide elution studies and crystallization of HLA-DR1 (Chicz et al., 1993; Brown et al., 1993). In contrast, the peptide binding site of a large fraction of the recombinant DR2 molecules is readily available for binding by a high affinity peptide. Recombinant DR2 molecules will therefore be useful for studying the peptide binding specificity of HLA-DR2 and for generating complexes with defined antigenic peptides.

**DISCUSSION**

Soluble HLA-DR1 has been expressed in the baculovirus system using cDNA constructs for the extracellular domains of DRα and DRβ. These molecules were assembled and secreted but had a tendency to aggregate unless they were loaded with a high affinity peptide (Stern and Wiley, 1992). Other MHC class II molecules (such as the product of the DRA, DRB5*0101 genes) showed a strong tendency to aggregate when this approach was attempted, even when high affinity peptides were added.2 The expression of mouse I-A molecules (I-Au and I-Ag7, which confer susceptibility to experimental autoimmune encephalomyelitis and to diabetes, respectively) has also been difficult. The native transmembrane region of these molecules could be replaced with a glycan-phosphatidyl inositol anchor; however, following cleavage of these molecules from the surface of transfected cells by phospholipase C, irreversible aggregation occurred even if the cells had been incubated with I-A binding peptides prior to cleavage.3

Soluble DR2 molecules could not be expressed in the Baculovirus system because the extracellular domains of DRα and DRβ chains did not assemble. These results indicated that the transmembrane regions were important for the proper assembly of DR2. Replacement of the hydrophobic transmembrane regions by the Fos-Jun heterodimer facilitates assembly and that it stabilizes the DR2 heterodimer.

The peptide binding site of MHC class II molecules is formed by the N-terminal domains of the MHC class II α and β chains; each chain contributes half of the floor and one of the two α-helices that flank the peptide binding site (Brown et al., 1993). The fact that the two chains have to be properly paired

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2 K. Vranovsky and J. L. Strominger, unpublished results.
3 L. Fugger and H. McDevitt, personal communication.
for the peptide binding domain to be formed may explain some of the difficulties that have been encountered with the expression of recombinant MHC class II molecules. This report demonstrates that a MHC class II molecule can be appropriately assembled and secreted by P. pastoris. This eucaryotic expression system has the advantage that yeast cell transformants; colonies appear 3–4 days following transformation and can be readily screened for integration of plasmid DNA by PCR. Positive colonies can be rapidly expanded in liquid media and tested for expression of the recombinant protein following induction of the AOX1 promoter. The AOX1 promoter has a low basal level of transcription; high level transcription can be rapidly induced by addition of methanol to the growth media (Cregg et al., 1987).

Some secreted proteins have been expressed at very high yields (>1 g/liter) in this system; the expression level of HLA-DR2 was lower (300–400 μg/liter), possibly because heterodimer assembly was the rate-limiting step. Availability of (low affinity) peptides in the secretory pathway may be the limiting factor in the assembly of DR αβ heterodimers; this may be overcome by coexpressing a DR-binding peptide in the secretory pathway (under the control of the AOX1 promoter as fusion with the α-mating factor secretion signal). The invariant chain peptide (CLIP) may be suitable for this purpose since it may be later exchanged with other DR2 binding peptide(s) (Avva and Cresswell, 1994).

Soluble DR2 molecules will allow a detailed characterization of the peptide binding specificity of HLA-DR2 in an effort to define the mechanisms by which this MHC molecule confers susceptibility to multiple sclerosis.

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4 Recent studies have demonstrated that DR2 molecules that carry the leucine zipper dimerization domains are expressed at 1–2 mg/liter in insect cells (Drosophila Schneider cells) (L. Gauthier and K. Wucherpfennig, unpublished results).

Note Added in Proof—Since submission of this manuscript for publication, the use of a leucine zipper dimerization motif for the expression of a murine MHC class II molecule (I-Aκ) has been reported (Scott et al., 1996).

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