Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9703–9706, October 1993
Immunology

The segment of invariant chain that is critical for association with major histocompatibility complex class II molecules contains the sequence of a peptide eluted from class II polypeptides

INA M. FREISEWINKEL*, KLAUS SCHENCK†‡, AND NORBERT KOCH*†

*Section of Immunobiology, Institute of Zoology, University of Bonn, Römerstrasse 164, D-53117 Bonn, Germany; and †Institute of Immunology and Genetics, German Cancer Research Center, Heidelberg, Germany

Communicated by Hans J. Müller-Eberhard, July 14, 1993 (received for review February 18, 1993)

ABSTRACT **Major histocompatibility complex class II molecules present peptides from an extracellular source of antigens to CD4+ T lymphocytes. The class II-associated invariant chain affects this role of α and β polypeptides by restriction of peptide loading to endocytic vesicles. Up to now no specific portion of the invariant chain has been defined as the class II binding site. We constructed recombinant invariant chain genes and inspected association of the mutant invariant chains with class II polypeptides. Here we demonstrate that an extracytoplasmic sequence of the invariant chain (aa 81–109) that is only 23 residues away from the transmembrane region is essential for contact with class II polypeptides, whereas the remaining C-terminal part is dispensable for binding. The sequence of invariant-chain-derived peptides that were eluted from class II molecules is contained in this segment and may define the class II binding site of the invariant chain. The membrane-proximal position of this region suggests that the invariant chain and invariant-chain-derived peptides isolated from class II molecules bind to a domain distinct from the class II pocket.**

Recently, it has been demonstrated that expression of the invariant chain (Ii) facilitates the presentation of various antigens by major histocompatibility complex (MHC) class II molecules (1–5). Ii is a type II membrane protein with the C-terminal domain expressed at the luminal side of the endoplasmic reticulum membrane (6). The following support for two roles of Ii was found. (*i*) Ii carries a signal sequence on its cytoplasmic domain that is responsible for sorting Ii and associated MHC class II molecules from the secretory to the endocytic route (7–9). (*ii*) Ii impedes loading of peptides to the MHC class II groove, a process that is postponed until MHC class II molecules enter an endocytic compartment and Ii is degraded (10–12). Additionally, assembly of Ii with MHC class II polypeptides modulates the shape of α and β dimers, which can promote the recognition of the αβ peptide complex by T cells (13, 14).

A soluble 18-kDa fragment of Ii that consists of most of the C-terminal part was found to bind to MHC class II molecules, indicating that association is not mediated by the membrane-spanning region or the cytoplasmic N terminus (15). Accordingly, a hybrid of the luminal domains of class II molecules and the transmembrane and cytoplasmic regions of MHC class I molecules associates with Ii (16). In this report we show that a membrane-proximal sequence of the luminal part of Ii is responsible for association with MHC class II molecules.

MATERIALS AND METHODS

Plasmid Constructions. A 9.4-kb fragment containing the murine Ii gene was subcloned into the plasmid pUC9 (1). Deletions of coding regions of the Ii gene were obtained by restriction enzyme digestion and religation. Noncorresponding sticky ends were filled-in with T4 polymerase and blunt-end-ligated. The deletion clones were characterized by restriction enzyme digestion and those with a recombination within the coding region were sequenced. In two of the clones, the recombination led to exchanges of one or two codons. The deletion mutant $Ii_{\Delta 192-212}$ contains an additional valine at position 192, and $Ii_{\Delta 126-215}$ was elongated by an isoleucine (position 126) and a lysine (position 127). aa 209–255 of $Ii_{\Delta 153-208}$ correspond to the sequence encoded by exon 6b. The Ii gene, the recombinant Ii constructs, and the HLA-DRα and HLA-DRβ3 cDNAs were subcloned into the pcEXV-3 expression vector. The 5′ sequences of Ii constructs were derived from the murine Ii31 cDNA (bp 1–93 of the coding region) and fused to the respective 3′ genomic sequences via the *Sac* I restriction enzyme recognition site within exon 2. For stable transfections, plasmids pCA69 and pCA39 carrying the IEα and -β genes were used. Plasmid pcEXV-3-mIi3 (Ii31 cDNA) was provided by Jim Miller (University of Chicago) and pCA39 and pCA69 were provided by Bernard Malissen (Centre d'Immunologie de Marseille-Luminy, France).

Cells and Transfections. For transient transfections, COS-1 cells that do not endogenously express Ii or MHC class II molecules were seeded at a density of 2×10^6 cells in a 100-mm dish 1 day prior to transfection. Transfections were carried out as described (17). The cells were transfected with 4 μg of recombinant Ii constructs subcloned into pcEXV-3 and 15 μg of pcEXV-3-DRα and -β cDNAs, respectively. The class II- and Ii-negative fibroblast cell line Rat2 was stably cotransfected with IE genes (pCA69 and pCA39), the recombinant murine gene encoding $Ii_{\Delta 81-127}$, or wild-type Ii in pUC9 and with the neomycin-resistance gene (pAG60) as described (18). Single cell clones were selected with G418.

Metabolic Radiolabeling and Immunoprecipitation. Cells were harvested 20 h after transfection and labeled for 15 min with [^{35}S]methionine at 37°C. Cells were lysed with 1% Nonidet P-40 in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride/0.024 Trypsin inhibitor units of aprotinin per ml). Ii polypeptides were immunoprecipitated with anti-Ii monoclonal antibody (mAb) In1, which is directed against the N terminus (19); HLA-DR molecules were immunoprecipitated with the mixture of anti-DR mAbs I251SB (20), ISCR3 (21), and 2.06 (22); and IE chains were immunoprecipitated with mAb K22-42 (23). In Figs. 2 and 3, lysates were precleared by incubation with Sepharose CL-4B and in Fig. 4 lysates were precleared by affinity chromatography on lentil lectin-Sepharose 4B columns.

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Abbreviations: MHC, major histocompatibility complex; CBS, class II binding site; Ii, invariant chain; mAb, monoclonal antibody.

‡Present address: Convatec GmbH, Volkratstrasse 83, D-80632 Munich, Germany.

Gel Electrophoresis. Immunoprecipitates were separated on 10–15% polyacrylamide gradient/SDS gels. In two-dimensional gels, proteins were separated according to their charge in the first dimension using nonequilibrium pH gradient gel electrophoresis and according to their molecular weight in the second dimension in 13% polyacrylamide gels.

RESULTS AND DISCUSSION

Expression of Recombinant Ii Polypeptides and Association with Class II Polypeptides. To identify the class II binding site (CBS) of Ii, we deleted four regions of the murine Ii gene that encode aa 192–212, aa 153–208, aa 110–161, or aa 81–127 of the extracytoplasmic domain. These deletions spanning almost the whole luminal part of the molecule are delineated in Fig. 1. Immunoprecipitates of the mutant Ii molecules from transiently transfected COS-1 cells are shown in Fig. 2*A*. The mobility shifts of the recombinant polypeptides in SDS gels are in agreement with the sizes of the deleted regions. To assess the ability of the recombinant Ii molecules to assemble with class II polypeptides, the deletion constructs and class II HLA-DRα and -β cDNAs were coexpressed in COS-1 cells. Association of the mutant Ii molecules with class II polypeptides was monitored by coimmunoprecipitation. Fig. 2*B* shows that $Ii_{\Delta 192-212}$, $Ii_{\Delta 153-208}$, and $Ii_{\Delta 110-161}$ were coprecipitated with class II polypeptides, suggesting that these recombinant Ii polypeptides contain a domain capable of associating with class II chains. In contrast, the recombinant $Ii_{\Delta 81-127}$ was not detected in class II immunoprecipitates (Fig. 2*B*, lane 7), although $Ii_{\Delta 81-127}$ was expressed in the transfectants and could be precipitated with an anti-Ii antibody (Fig. 2*B*, lane 8). These experiments were repeated several times and were always consistent. In concert with this result, no association of $Ii_{\Delta 81-127}$ with murine class II molecules could be detected in Rat2 fibroblasts after stable cotransfection of the $Ii_{\Delta 81-127}$ construct and IE genes. $Ii_{\Delta 81-127}$ was not coprecipitated with IE polypeptides (Fig. 3*A*), even though it was synthesized in strong excess over class II α and β chains. Vice versa, class II polypeptides were not detected in anti-Ii immunoprecipitates (Fig. 3*B*). Coprecipitation of wild-type Ii and IE genes from stably cotransfected Rat2 fibroblasts demonstrated that the assembly of class II and Ii molecules in these fibroblast cells was not affected (Fig. 3*C*). Stable transfections with other deletion constructs confirmed the results of Fig. 2 (data not shown).

The C-Terminal Part of Ii Containing aa 126–215 Is Dispensable for Binding of Class II Molecules. The aa 110–161 deletion partially overlaps aa 81–127. Thus, coprecipitation of $Ii_{\Delta 110-161}$ with DR molecules suggests that the deletion still allowing association with class II can be extended to the border of aa 109. Coisolation of a recombinant $Ii_{\Delta 110-130}$ (see Fig. 1) with class II molecules confirms this result and confines the class II binding domain to a sequence encoded predominantly by exon 3 of the Ii gene (Fig. 4). The sequences encoded by exons 4–8 should then be dispensable for binding of class II molecules. To prove this, we opposed $Ii_{\Delta 81-127}$ to a recombinant Ii in which the residual C terminus with aa 126–215 was truncated (see Fig. 1). Fig. 4 shows that $Ii_{\Delta 126-215}$ is present in class II immunoprecipitates of transfected COS-1 cells. This result indicates that in $Ii_{\Delta 126-215}$ the CBS is preserved. In conjunction with the results shown above, it can be concluded that the 106 C-terminal amino acids of Ii have no significant effect on the association with class II polypeptides.

The Sequence of an Ii-Derived Peptide (aa 85–99) That Was Eluted from Class II Molecules Is Highly Conserved in Mice, Rats, and Humans. The sequence of murine Ii aa 81–109, which is essential for class II binding, was aligned with sequences from rat and human Ii polypeptides (Fig. 5). A substantial part of these sequences is identical. This high degree of conservation could explain promiscuous binding of murine and human Ii to class II molecules from both species (29).

Recently, it was reported that a number of peptides that were eluted from various class II types contain one set of peptides that is derived from Ii (27, 28, 30). It was interpreted that these peptides bind to the class II pocket, since their sequences share some anchor residues with other class II-eluted peptides (28, 31) and, additionally, were described to confer resistance to SDS-induced $\alpha\beta$-chain dissociation (28). Ii-derived peptides eluted from either human or murine class II molecules possess sequences that are centered to the highly conserved part of the region we found indispensable for association (Fig. 5), suggesting that these residues of Ii are engaged in interaction with class II molecules. Our data argue that the Ii peptide isolated from class II molecules is a degradation fragment of Ii and remains bound to class II molecules at a position distinct from the peptide-binding groove. This is also supported by the recent finding that incubation of $\alpha\beta$ dimers with the Ii peptide of aa 81–104 that contains the CBS does not confer SDS-resistance to class II

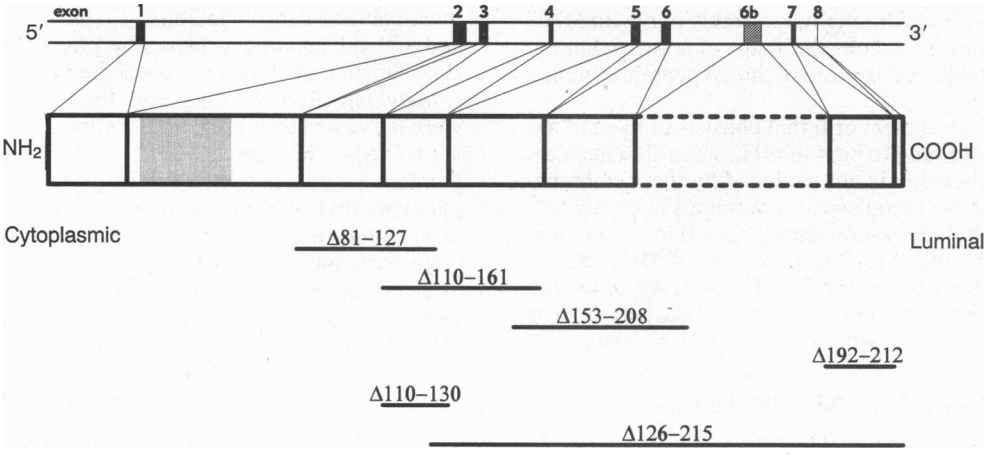

FIG. 1. Schematic representation of recombinant Ii polypeptides encoded by deletion mutants of the Ii gene. The Ii gene consists of exons 1–8 (solid boxes), which encode the predominant invariant chain form Ii31. An additional coding region, exon 6b (hatched box), is alternatively spliced yielding a 41-kDa polypeptide containing an extra domain of 64 aa (dotted lines) (24). The transmembrane region is represented by a lightly shaded box. The respective deletions of the mutant Ii polypeptides are delineated below the diagram. Deleted amino acids are numbered according to Ii31 with exception of $Ii_{\Delta 153-208}$, in which a sequence at the end of exon 5 is fused to a sequence within exon 6b.

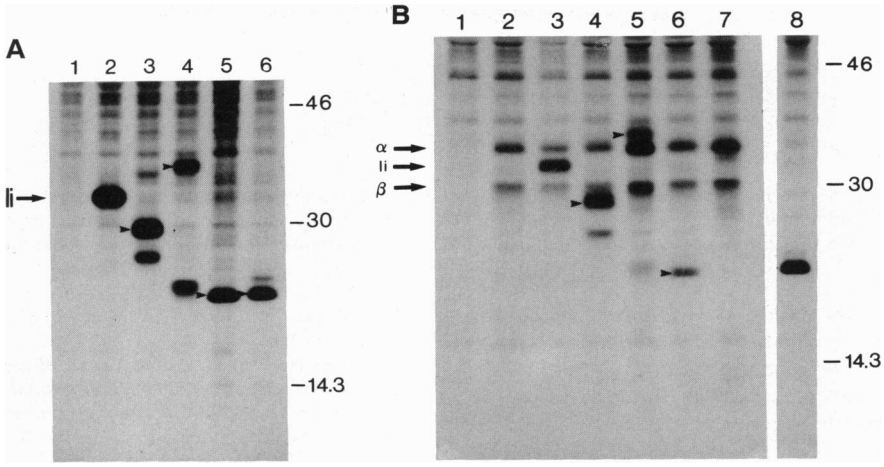

FIG. 2. (*A*) SDS/PAGE of recombinant polypeptides expressed in transiently transfected COS-1 cells and immunoprecipitated with mAb In1. The mutant Ii molecules were characterized on the basis of their molecular masses. Lanes: 1, vector-transfected control; 2, Ii; 3, $Ii_{\Delta 192-212}$ (29 kDa); 4, $Ii_{\Delta 153-208}$ (35 kDa); 5, $Ii_{\Delta 110-161}$ (19 kDa); 6, $Ii_{\Delta 81-127}$ (20 kDa). The estimated molecular masses of the recombinant Ii molecules separated in lanes 3–6 are shown in parentheses. (*B*) SDS/PAGE of anti-DR immunoprecipitates from COS-1 cells transiently transfected with the respective Ii constructs and the DRα and DRβ cDNAs. Lanes: 1, vector-transfected control; 2, α and β; 3, Ii, α, and β; 4, $Ii_{\Delta 192-212}$, α, and β; 5, $Ii_{\Delta 153-208}$, α, and β; 6, $Ii_{\Delta 110-161}$, α, and β; 7, $Ii_{\Delta 81-127}$, α, and β. Lane 8 shows an anti-Ii immunoprecipitate of the transfection shown in lane 7. $Ii_{\Delta 192-212}$ and $Ii_{\Delta 153-208}$ are partially degraded upon cell lysis and thus degradation bands are present. $Ii_{\Delta 153-208}$ is derived from the 41-kDa form of Ii and is, therefore, larger than Ii. In COS-1 cells, expression of the alternatively spliced 41-kDa form of Ii appears to be drastically reduced. Positions of Ii31, DRα, and DRβ are indicated on the left. The migration of molecular mass markers is shown on the right in kDa. Arrowheads mark positions of recombinant Ii polypeptides.

polypeptides (32). Since it has been demonstrated that this peptide blocks the formation of compact DR molecules (32), the interaction of class II with Ii solely at the CBS is probably sufficient to protect the class II pocket from binding of antigenic peptides.

The membrane-proximal location of the CBS of Ii suggests that the second immunoglobulin-like domain of class II polypeptides associates with Ii. This is consistent with a result showing that Ii associates with a site different from staphylococcal enterotoxin, which binds to a position in the first domain of class II molecules (33).

At present three functional segments of Ii have been defined. The signal sequence for targeting Ii to endosomes is encoded by exon 1 of the Ii gene and the membrane-spanning region is encoded by a sequence in exon 2 (7, 8, 24). In this report we show that amino acid residues encoded by exon 3 are responsible for association with class II polypeptides. Since these three protein segments are contained in not more than half of the Ii polypeptide, it is tempting to speculate what functional properties of Ii are carried by the remaining part of

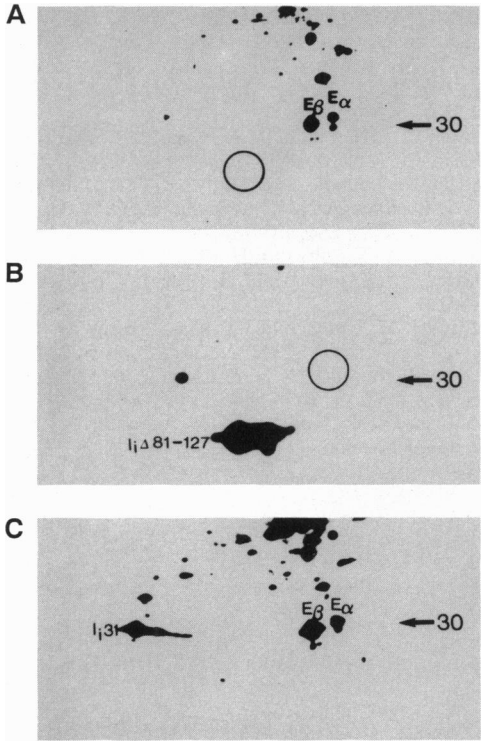

FIG. 3. Two-dimensional separation (nonequilibrium pH gradient gel electrophoresis in the first and SDS/PAGE in the second dimension) of anti-IE immunoprecipitates (*A* and *C*) and anti-Ii immunoprecipitates (*B*) after stable transfection of Rat2 fibroblasts with IE genes and either $Ii_{\Delta 81-127}$ (*A* and *B*) or wild-type Ii (*C*). E_α, E_β, and Ii31 indicate IE chains and the 31-kDa form of wild-type Ii, respectively. Circles denote the positions of the 31-kDa derivative of the $Ii_{\Delta 81-127}$ chain (*A*) or IE chains (*B*), respectively, where they usually appear. Migration of a molecular mass standard is marked on the right in kDa.

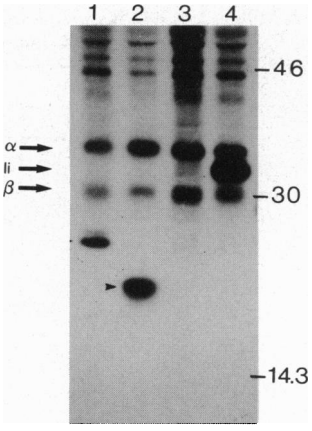

FIG. 4. SDS/PAGE of anti-DR immunoprecipitates from COS-1 cells transfected with the recombinant $Ii_{\Delta 110-130}$ and $Ii_{\Delta 126-215}$ constructs and the DRα and DRβ cDNAs. Lanes: 1, $Ii_{\Delta 110-130}$, α, and β; 2, $Ii_{\Delta 126-215}$, α, and β; 3, α and β; 4, Ii, α, and β. Positions of Ii31, DRα, and DRβ are indicated on the left. The migration of molecular mass markers is delineated on the right in kDa. Arrowheads show positions of recombinant Ii polypeptides.

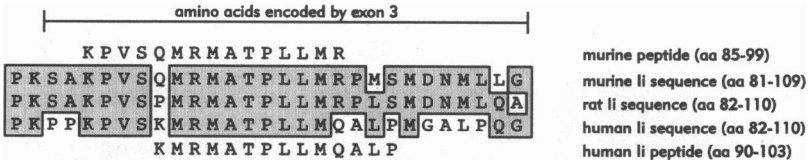

FIG. 5. Comparison of the murine Ii sequence that contains the CBS with homologous sequences of rat and human Ii. The amino acid sequences of murine, rat, and human Ii (6, 25, 26) were aligned and compared with the sequences of the shortest peptides isolated from murine and human class II molecules (27, 28). Conserved amino acids are boxed. Amino acids encoded by exon 3 of the murine Ii gene are indicated at the top.

the molecule encoded by exons 4–8 of the Ii gene. The C-terminal portion that could have the steric potential to interact with the peptide-binding groove does not intimately associate with class II dimers. However, in the 41-kDa form of Ii, this part contains the sequence encoded by the alternatively spliced exon 6b (24, 34), which suggests that this domain carries other functional properties not yet known.

We thank C. Voßhenrich and A. König for technical assistance, H. Kanzler and G. Siebenkotten for critically reading the manuscript, and M. Schlich for photography. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ko 810/4) and by the Sonderforschungsbereich (284).

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