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*J Immunol* 2001; 166:3309-3314; doi: 10.4049/jimmunol.166.5.3309
http://www.jimmunol.org/content/166/5/3309

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Association of Mouse Mammary Tumor Virus Superantigen with MHC Class II During Biosynthesis

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Mouse mammary tumor viruses encode superantigens that interact with MHC class II proteins and stimulate T cells. We show here that presentation of mouse mammary tumor virus superantigen does not require DM. Furthermore, we have identified a strong class II peptide binding motif in the Mtv-7 superantigen, and we show that this motif is necessary for association with class II molecules in vitro translation and in vivo functional assays. Our results suggest that endogenously synthesized viral superantigen can bind to MHC class II heterodimers during biosynthesis in the endoplasmic reticulum in a manner analogous to that used by the class II-associated invariant chain. The Journal of Immunology, 2001, 166: 3309–3314.

Materials and Methods

Constructs and plasmids

HLA-DR1α (12) and HLA-DRβ1 (13) cDNAs were cloned into pSP72 (Promega, Madison, WI) behind the T7 RNA polymerase promoter (14). In the Ii/Mtv-7 sag constructs, IM1 and IM2, the amino-terminal portion of the Mtv-7 sag was replaced with the corresponding region of the Ii CDNA. IM1 contains an Ii CDNA replacement, corresponding to aa 1–106, which includes the CLIP motif (MRMATPLLM), while construct IM2 contains an Ii cDNA replacement, corresponding to aa 1–90, excluding the CLIP region. Both constructs contain the Mtv-7 sag extracellular portion from the transmembrane domain, including the CIIPBM (YNLNNSENS). Construct m86IM2 is derived from IM2 by altering the position 1 (P1) and P2 positions of CIIPBM by site-directed mutagenesis, Y3A and N3A.

Pep tide scoring system

The peptide scoring system reflects the relative capacity of nonameric peptides to bind to MHC class II molecules (17). It is based on the hypothesis that this interaction is a result of all side chain effects in any given peptide, and that the magnitude of the side chain effect of a particular amino acid

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0022-1767/01/$02.00
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depends on its relative position with regard to the anchor at P1. Because the P1 anchor requires an amino acid with either a long aliphatic or an aromatic side chain to interact with the deep pocket in the class II peptide binding groove, only seven amino acids (F, I, L, M, V, W, and Y) can be considered for this position. Thus, the protein (Mtv-7 sag, li, or influenza virus hemagglutinin 306–318 peptide) (11) was scanned for all potential P1 anchors, and then the adjacent 8 aa residues (P2–P9) were located, and values obtained from side chain scanning on DRβ1*0401 as defined by the algorithm (17) were assigned to each amino acid. residue of the selected nonamer. The sum of these values was the peptide score. A score of 2 or more predicted class II binding.

In vitro translation and immunoprecipitation

The cDNAs were transcribed in vitro, either together or separately, using T7 RNA polymerase (Promega). The optimal amount of RNA for translation was determined empirically for each separate batch of RNA. In vitro translations were performed with rabbit reticulocyte lysate, not supplemented with DTT (Flexi; Promega), in the presence of canine pancreatic microsomal membranes (Promega). Translations were performed for 60 min at 30°C. After translation, microsomes were pelleted by centrifugation at 14,000 rpm for 10 min at 4°C and subsequently lysed in 200 μl of Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), and 5 mM MgCl2). Solubilized microsomes were subjected to immunoprecipitations. Nonspecifically bound proteins were removed by preclearing twice with 3 μl of normal rabbit serum or mouse ascites for 45 min with 50 μl of protein G beads (Immunopure immobilized protein G beads; Pierce, Rockford, IL). Immunoprecipitations with mAbs T366 or P1-1 were performed for 1 h on a shaker at 4°C. Immune complexes were removed by adsorption onto 50 μl of protein G beads. Pelleted protein G beads were washed four times in wash buffer, containing 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA. Bound immune complexes were eluted in reducing SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-ME, and 4% SDS) for 5 min at 95°C and subjected to SDS-PAGE.

Transfection and T cell stimulation assay

DRB1*0301 DMB wild-type and mutant human B-LCL (18) were transfected with the Mtv-7 sag gene under control of the human β-actin promoter (15). Stable transfected were screened for the level of DRB1*0301 expression by immunofluorescence and, and matched clones were used for the functional stimulation of the Vβ6+ T cell hybrid, RG17 (19), at various responder to stimulator ratios, T cell stimulation was measured by the level of IL-2 production, assessed in a bioassay by the proliferation of the IL-2-dependent cell line, HT-2. Transfected cells were cultured in complete medium at 5 × 105/ml in the presence of G418 (1 mg/ml). The expression of Mtv-7 sag mRNA in stable transfected was monitored by Northern blotting. FACS analysis with anti-HLA DR mAb (L243; BD Pharmingen, San Diego, CA) was performed to assay the expression of DRB1*0301 in both the transfected wild-type (8,16) and DMB mutant (9,5,3) cells. The mean fluorescence intensity of DRB1*0301 expression in each cell line was between 98 and 102. Stable transfected were washed and mixed with 2 × 105 Vβ6+ RG17 T cell hybrids in 0.4 ml of complete medium and incubated for 24 h at 37°C. Plates were then frozen, and 100 μl of thawed supernatant was tested in quadruplicate for IL-2 concentration using 2 × 105 HT-2 cells/well in a 96-well round-bottom plate. Proliferation of HT-2 cells was measured by incorporation of [3H]thymidine (1 μCi/well). Chinese hamster ovary cells transfected with murine Eβ3, CHIC1Ta (20) were transfected with wild-type Mtv-7 sag (CHIC/Eβ3) (20) or the P17 mutant gene under control of the human β-actin promoter phβαA-P17ST, using Lipofectamine (Life Technologies, Gaithersburg, MD). Two days after transfection, the cells were trypsinized, washed, and plated at 10, 102, or 103 cells/well in flat-bottom 96-well plates in the presence of G418 (1 mg/ml) and puromycin (25 μg/ml) (Sigma, St. Louis, MO). Stable transfected were selected for expression of P17ST by Northern blotting and for Eβ3 by FACS analysis using mAb 14.4.4s-FTIC (BD Pharmingen, San Diego, CA). Clones expressing high levels of both P17ST and Eβ3 were tested for T cell stimulation using an equal number of the Vβ6+ T cell hybridoma Oms (20) (2 × 105/well; stimulus/responder ratio, 1:1). IL-2 production was measured by HT-2 cell proliferation.

Results and Discussion

MMTV superantigen expression does not require DM

Class II molecules bind peptides generated by proteolysis in the endocytic pathway (1). Before the class II dimers can be loaded with peptides, li must be proteolytically destroyed, and CLIP needs to be dislodged from the peptide binding groove. CLIP removal and subsequent peptide loading are catalyzed by a class II-like molecule, DM (5). Cell lines deficient in DM have a reduced capacity to present externally added protein Ags, while no defect is seen in the presentation of short synthetic peptides (5, 6). We show here that presentation of the Mtv-7 superantigen by class II, as measured by T cell recognition, does not require DM. The DRB1*0301-expressing DMB mutant (9,5,3) and wild-type (8,1,6) B lymphoblastoid cell lines (18) were transfected with Mtv-7 sag (15). Stable transfected, matched for the level of DRB1*0301 on their cell surface and Mtv-7 sag mRNA (data not shown), were assayed for superantigen expression by their capacity to stimulate the Vβ6+ T cell hybrid, RG17 (19). No difference in stimulatory capacity was seen between DMB wild-type and mutant cells (Fig. 1). The phenotype of the DMB mutant cell line was confirmed by staining with an anti-CLIP mAb, 1-5, which recognizes class II molecules only from DM mutants, but not from wild-type cells (data not shown). If presentation of the Mtv-7 superantigen were due to proteolytic processing of the Ag in the endocytic pathway, the efficacy of presentation would be compromised by the absence of DM. Because no such effect was observed, our results are consistent with the idea that the Mtv-7 superantigen is presented intact. These results raise the question of how a protein synthesized by the APC itself can associate with MHC class II and be presented on the cell surface. Furthermore, in which cellular compartment does the MMTV superantigen associate with class II? We postulate that the association of MMTV superantigen with MHC class II occurs in the same manner as the interaction of class II with li.

CILPBM in the Mtv-7 superantigen

Both Mtv-7 superantigen and li are type II transmembrane glycoproteins of similar size (Fig. 2A) (16, 21), and both are known to associate with the MHC class II β2m heterodimer. The association of li and MHC class II involves the class II peptide binding groove and takes place in the ER via the CLIP region of li (10, 11, 22). We scanned the Mtv-7 superantigen amino acid sequence for a motif that fulfills the requirements for class II binding, applying the side chain scoring algorithm developed by Hammer et al. (17). The peptide score, derived from the sum of corresponding side chain values of the quantitative motif, has to be ≥2 for a peptide to be considered to have binding affinity for MHC class II. The region spanning aa 86–95 of the Mtv-7 superantigen (YNLNNSENS) exhibits a high class II binding score of 5.8 (Fig. 2A). Interestingly,
this CIIPBM is conserved among various MMTV superantigens and occurs at a location similar to that of CLIP in intact Ii (Fig. 2A). In comparison, the Ii CLIP (MRMATPLLM) which has no amino acid identities with CIIPBM defined in the Mtv-7 superantigen, has a score of 3.9. Similarly, the influenza virus HA peptide 308–316, which was determined by x-ray crystallography to bind HLA DR1 in an identical manner to that in which Ii CLIP binds HLA DR3 (11), was predicted to have a binding score of 4.3. The
presence of a CIIPBM in the Mtv-7 superantigen led us to hypothesize that the MMTV superantigen may be structurally analogous to Ii and thus associate with MHC class II in a similar manner.

Association of MMTV superantigen with DR1 αβ heterodimer in an in vitro translation system

To investigate possible interaction of the Mtv-7 superantigen and class II molecules, we used an in vitro translation system, previously shown to support the early steps of class II assembly (14). Class II αβ dimers, generated by in vitro translation in rabbit reticulocyte lysate in the presence of microsomes, are indistinguishable from those found in living cells in terms of their ability to associate with intact Ii or bind peptide. Because the in vitro assembly of DR1 αβ dimers has been described in detail (14), we chose this class II substrate to explore a possible interaction with the Mtv-7 sag product. This superantigen is presented equally well by HLA-DR1 and by mouse class II I-E molecules to murine Vβ6+ T cell hybrids (23) and human T cells (24). Two chimeric constructs, IM1 and IM2, consisting of the cytoplasmic and transmembrane domains of Ii and the extracellular C-terminal domain of Mtv-7 sag (see Fig. 2A), were generated for use by in vitro translation with DR1 α- and β-chains. These chimeric molecules can be recognized by the mAb Pin-1, directed against the N terminus of Ii (25). IM1 includes the N-terminal portion of Ii, aa 1–106, which contains CLIP, while IM2 has a similar truncated portion of Ii, aa 1–90, that excludes the CLIP region. In vitro translation studies of truncated Ii chain with HLA-DR1 α- and β-chains have demonstrated that the CLIP region between aa 96 and 104 is critical for association with class II, as deletion of this region abolished binding (10).

The cDNAs encoding IM1, IM2, and HLA-DR1 α- and β-chains were used for the production of mRNAs that were translated, either separately or in combination, in the presence of canine pancreatic microsomal membranes. Immunoprecipitations were performed on detergent extracts from these microsomes with an mAb against the assembled DR1 complex, Tu36 (26), or against the cytoplasmic tail of Ii, Pin-1 (25). The IM1 and IM2 translation products can be recognized by mAb Pin-1, but do not react with either mAb Tu36 or rabbit anti-HLA DRα and anti-HLA DRβ polyclonal Abs (data not shown). When mRNAs of IM1 or IM2
were cotranslated with DR1α and -β mRNAs in vitro, immunoprecipitation with Tu36 resulted in recovery of αβ dimers, as well as αβ/IM1 and αβ/IM2 complexes, but not IM1 or IM2 alone (Fig. 2B). In reciprocal fashion, Pin-1 immunoprecipitated the IM1 and IM2 translation products as well as αβ/IM1 and αβ/IM2 complexes, but not αβ dimers alone (Fig. 2B). Thus, IM1 and IM2 can associate with the newly synthesized DR1 αβ heterodimer. The IM1 construct served as a positive control in these experiments, because it contains the Ii-derived CLIP region. The fact that IM2, which lacks CLIP, associated with DR1 as well as IM1 indicates that CIIPBM in the superantigen may contribute to the observed binding. IM2 did not associate with either free α- or β-chain of class II when cotranslated in vitro. This suggests that formation of the αβ/IM2 complexes can occur only through an αβ dimer intermediate (data not shown).

To confirm the role of the class II binding motif in the association of the MMTV superantigen with class II molecules, we changed the P1 and P2 positions from tyrosine and asparagine to alanine and glycine, respectively (Fig. 2A), destroying the anchor position of the peptide binding motif. As predicted, the ability of this mutant, m86IM2, to associate with the DR1 αβ heterodimers was dramatically reduced in the in vitro translation reaction (Fig. 2C). These data suggest that the CIIPBM region involved in the association between MMTV superantigen and MHC class II is analogous to that of Ii CLIP with class II. Competitive inhibition studies indicated that Ii CLIP bound with higher affinity to HLA DR1 than the CIIPBM, while a negative control peptide had no influence on the interaction between Mtv-7 and DR1 (data not shown). The C-terminal domain of recombinant soluble Ii protein forms an α-helix that trimerizes and is able to interact with empty class II molecules in the ER. It has been suggested that this trimerization augments the affinity of the Ii chain for class II αβ dimers (27). No trimer or oligomer formation of the MMTV superantigen could be detected in the in vitro translation system under conditions in which Ii trimers are readily seen (data not shown) (10). This lack of trimer formation offers a possible explanation for findings in Ii knockout mice (28), in which the MMTV superantigen was unable to override the Ii defect, as judged by the level of MHC class II expression and CD4+ T cell development. It is possible that the synthesis rate of the Mtv-7 sag is much slower than that of Ii in vivo.

**Mutation in Mtv-7sag CLIP affects functional superantigen expression**

To further confirm the importance of the class II binding motif in the Mtv-7 superantigen, we introduced the m86 mutation in the intact Mtv-7 sag gene, referred to as P1S7. This was subcloned into the eukaryotic expression vector phAcp;I.neo and transfected into Chinese hamster ovary cells expressing mouse I-Eα, CHIE cells (20). Stable transfectants were derived and tested for mutant P1S7 mRNA by Northern analysis and for levels of class II expression by staining with the anti-I-Eα mAb 14.4.4s. Clones with matched expression (Fig. 3, A and B) were tested for superantigen expression by stimulation of the Vβ6+ T cell hybrid, Omis (20), compared with wild-type Mtv-7 sag-transfected CHIE cells, CHIE/S7 (20) (Fig. 3C). T cell stimulation was measured by the level of IL-2 production, as detected by proliferation of the IL-2-dependent cell line, HT-2. The mutation in the Mtv-7 sag CIIPBM negatively affected functional presentation of superantigen to T cells (Fig. 3C), suggesting that the mutation inhibits superantigen presentation at the cell surface.

**Model of MMTV superantigen association with class II**

Previous biochemical studies by Winslow et al. have shown that the Mtv-7 superantigen is synthesized in the ER as a 45-kDa glycoprotein (gp45), while an 18-kDa carboxyl-terminal fragment is detected at the cell surface (29). Because gp45 is by far the predominant form detected in immunoblot analyses, most Mtv-7 superantigen appears to be retained and degraded in the ER. It is presently unclear how the MMTV superantigen leaves the ER, but it is possible that the limiting factor is the inefficiency with which MMTV competes with Ii for binding to the MHC class II heterodimer during assembly. MMTV superantigen is not detected on the surface of B lymphocytes of inappropriate MHC class II haplotypes, as assessed by immunohistochemical and immunofluorescent techniques (29), implying that MMTV superantigen presentation in vivo requires the appropriately matched class II product. Because mice that lack Ii show reduced level of MMTV superantigen activity (28), Ii could play a role in the trafficking of the MMTV superantigen/class II complex, perhaps by forming mixed trimers, composed of Ii and superantigen. Mixed oligomers of the p31 and p41 isoforms of Ii, the latter a splice variant of Ii that contains an additional 80 residues, have been described and demonstrate the ability of Ii to engage in mixed oligomer formation (30). Such mixed complexes may be able to exit the ER and follow the MHC class II maturation pathway through Golgi/post-Golgi and endocytic compartments (Fig. 4). The trans-Golgi network is the site where furin, a protease previously implicated in selective proteolytic processing of the Mtv-7 superantigen (29), is located. Based on the intracellular distribution of furin, its action precedes that of the proteases in the endocytic pathway, which might also act on the MMTV superantigen (see model in Fig. 4). This hypothesis fits with the observation that no full-length membrane form of the MMTV superantigen has been detected at the cell surface, whereas an 18-kDa carboxyl-terminal fragment remains associated with the class II protein on the cell surface (29). Thus, the CIIPBM might be necessary, but not sufficient, for binding, because the 18-kDa fragment might presumably also contribute to binding.

These data indicate that MMTV superantigen interacts with class II via a CIIPBM in its luminal domain with the peptide binding cleft, analogous to Ii. This interaction positions the superantigen T cell epitope outside of the peptide binding groove, abrogating the requirement for DM. Therefore, we conclude that presentation of MMTV superantigen is different from the mode of presentation of conventional peptide Ag by MHC class II and may, in fact, imitate the mode of interaction of Ii with class II.

**Acknowledgments**

We thank Elizabeth Mellins for the gift of the DR3/DMB mutant cell lines and 1-5 mAb, Marie-Jose Bijlmakers for her initial help in setting up the in vitro translation studies, and Andrew Knight for the gift of EMCV promoter-driven expression vector. Gary Winslow generously provided the CHIE beige/CHIE/S7, and Omis cell lines, and Peter Cresswell kindly donated the Pin-1 mAb. Lia Kim provided valuable technical assistance.

**References**

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