Regions of Mouse Mammary Tumor Virus Superantigen Involved in Interaction with the Major Histocompatibility Complex
Class II I-A Molecule

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Mouse mammary tumor virus (MMTV) encodes a superantigen (SAg). SAgS are presented by major histocompatibility complex (MHC) class II molecules at the cell surface and interact with subsets of T cells expressing specific variable domains of the T-cell receptor β chain. MMTV SAgS can be divided into two groups, depending on their responses induced in I-E$^+$ and I-E$^-$ mouse strains (reviewed in reference 8). One SAg group requires the expression of I-E for presentation (e.g., GR, C3H, SIM, and Tes2 and mtv-1, -6, -8, and -9), whereas the other can be presented by either I-A or I-E (e.g., mtv-7, SW, SHN, Tes14, CS, and C4). The structural features conferring this difference remain unclear. We therefore studied hybrid molecules between the I-E-dependent GR and the I-E-independent mtv-7 SAgS for their abilities to be presented by I-A by comparing their SAg responses in I-E$^+$ I-A$^+$ and I-E$^-$ I-A$^-$ mice. Recombinant vaccinia virus (rVV) was chosen as a heterologous SAg expression system in vivo. In addition to the already described rVV expressing the I-E-dependent SAg of MMTV(GR) (vOGR1 [5, 6]), herein called v-GR, we produced a new rVV for the I-E-independent SAg of mtv-7 (v-7) (Fig. 1). To map sequences involved in I-A interaction, we further constructed rVVs for hybrid molecules between MMTV(GR) and mtv-7 SAgS. Since the comparison of protein sequences from I-E-dependent (GR, C3H, SIM, and Tes2 and mtv-1, -6, -8, and -9) and -independent viruses (mtv-7, SW, SHN, Tes14, CS, and C4) did not reveal any possible consensus site for MHC class II I-A binding, we divided the extracellular part of the MMTV SAgS into thirds and produced six rVVs expressing hybrid SAgS with all combinations of them (Fig. 2A, v-7G1 to v-7G6). The overall expression of the viral SAg by the rVVs was evaluated by immunoprecipitation of total lysates of CV1 cells (Fig. 2B). vv wild-type-infected cells were used as a negative control. Two major bands and one minor band were seen for each rVV around the size of 46 kDa, as previously described (5). v-7G3, -7G4, and -7G6 produced bands similar to the parental v-7, whereas v-7G1, -7G2, and -7G5 displayed bands with the size of the parental v-GR. Addition of tunicamycin during 35S labeling yielded a single 37-kDa band, corresponding to the nonglycosylated core protein (data not shown). Thus, the different banding was due to differential glycosylation in the extracellular, membrane-proximal third of the mtv-7 and GR SAgS. SAg bands were weaker for v-7, v-7G2, and v-7G6 and especially v-7G1 than for v-7G3, -7G4, -7G5, and v-GR. Despite the different intensity of their bands, v-7 and v-GR induced similar stimulation of specific CD4$^+$ T cells (Fig. 3). Since the different intensity of the bands strictly segregated with the C-terminal third of the SAgS, it was most likely caused by the anti-SAg antiserum recognizing the mtv-7 SAg less efficiently than the GR SAg. Indeed, this anti-SAg antiserum (serum C [3]) was raised against a synthetic 23-amino-acid (aa) peptide of the GR SAg (aa 262 to 284), which has a 3-aa difference with the corresponding mtv-7 SAg peptide. We concluded that our rVVs express a SAg of the appropriate size at similar levels in tissue culture, except for v-7G1, which exhibits a slightly weaker expression.

All rVVs were subsequently tested in I-E$^+$ I-A$^+$ (BALB/c or C57BL/6 I-E$^+$ tg) and I-E$^-$.I-A$^+$ (C57BL/6) mice under previously determined optimal conditions (data not shown). One day after infection with 10$^6$ PFU, draining popliteal lymph nodes were analyzed for the expansion of mtv-7-specific Vβ6$^+$ T cells and GR-specific Vβ14$^+$ T cells by immunofluorescence staining followed by flow cytometry. Cumulative results of all performed experiments, expressed as the percentage of Vβ$^+$ CD4$^+$ T cells of normal uninfected mice, are presented in Fig. 3. In I-E$^-$ mice, parental and hybrid SAgS efficiently stimulated CD4$^+$ T cells with the Vβ6 or Vβ14 T-cell receptor specificity predicted from their C-terminal 30 aa (Fig. 3A and C). rVVs v-7G1, -7G2, and -7G6 stimulated Vβ6$^+$ CD4$^+$ T cells in a manner similar to that of the control rVV v-7. v-7G1 induced a slightly but significantly lower response, in agreement with the lower SAg expression observed by immunoprecipitation (Fig. 2B). The Vβ14-specific rVVs v-7G3, -7G4, and -7G5 also...
FIG. 1. Comparison of amino acid sequences of the I-E-independent mtv-7 SAg and the I-E-dependent MMTV(GR) SAg. Asterisks indicate amino acid identity with the mtv-7 sequence. Dashes indicate gaps introduced to maximize amino acid identity. Potential N-linked glycosylation sites are marked with boxes. Grey boxes denote basic clusters that are potential recognition sites for convertases. Amino acids of the putative transmembrane domain (TM, aa 45 to 67) are marked with an arrow above the aligned sequence. Vertical arrows at positions 122 and 211 indicate the exchange points between mtv-7 and MMTV(GR) SAg's used for the creation of hybrid molecules.

| SAg mtv7 | MPRLQKWLNSRECPTLRRERAAKGLFPTKDDPSACTRMSPSDIDLILCCKLGIALCGL 60 |
| SAg GR  | ****************************************G**********HK************60 |
| SAg mtv7 | LGLGEAVRARRALFLDSIENSNSVQDYNNINNSNSSPLGCCQPTSSYKHPFPCEIE 119 |
| SAg GR  | ******************************************MN*** 120 |
| SAg mtv7 | IRMLAKNYPFTKNSIPGRRLILNAMESLPSFSTIFAFTQIRLEMGIS2NKRRTATVEQV 179 |
| SAg GR  | ****************************************K*** 180 |
| SAg mtv7 | QGLSATGLVEVKRSVVPVRDRWQPSTQYRIPYRDFALDTLNPGRDYLDNFPYWTV 239 |
| SAg GR  | ****************************************S**I*** 240 |
| SAg mtv7 | NGYKVLYRSLPPRLARAPRRNVPVCVLVTQEEDMKQVHPYIYLGTMNFWGKIFDYTE 299 |
| SAg GR  | ****************************************H**E*** 299 |
| SAg mtv7 | GAIAKILYNMKYTHGGGRVGFPRF 322 |
| SAg GR  | ******************************************MK**** 320 |

FIG. 1. Scheme of the different SAg's constructed and their expression in mammalian cells infected with rVV's. (A) The parental mtv-7 and MMTV(GR) SAg's and the hybrid mtv-7/GK SAg's constructed and used to produce rVV are shown. Only the extracellular C-terminal part of the viral SAg's is presented. TM indicates the putative transmembrane domain. Coding sequences for parental and hybrid SAg's were introduced into the VV transfer vector pARO1 under the control of the VV early p7.5K promoter. After sequencing, recombinant plasmids were used to generate rVV (WR strain) as described earlier (2, 5). Cells were lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris-HCl, pH 8) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, and 10 μg of leupeptin/ml) and were clarified by centrifugation. All samples were normalized for [35S] counts per minute before preclearing with preimmune rabbit serum and protein G-agarose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). MMTV SAg's were then immunoprecipitated with a polyclonal rabbit anti-MMTV(GR) peptide serum (serum C [3]) and protein-G agarose beads. Samples were resolved on a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis under reducing conditions. A VV wild-type-infected sample (wt) was used as a negative control.

FIG. 2. Scheme of the different SAg's constructed and their expression in mammalian cells infected with rVV's. (A) The parental mtv-7 and MMTV(GR) SAg's and the hybrid mtv-7/GK SAg's constructed and used to produce rVV are shown. Only the extracellular C-terminal part of the viral SAg's is presented. TM indicates the putative transmembrane domain. Coding sequences for parental and hybrid SAg's were introduced into the VV transfer vector pARO1 under the control of the VV early p7.5K promoter. After sequencing, recombinant plasmids were used to generate rVV (WR strain) as described earlier (2, 5). Cells were lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris-HCl, pH 8) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, and 10 μg of leupeptin/ml) and were clarified by centrifugation. All samples were normalized for [35S] counts per minute before preclearing with preimmune rabbit serum and protein G-agarose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). MMTV SAg's were then immunoprecipitated with a polyclonal rabbit anti-MMTV(GR) peptide serum (serum C [3]) and protein-G agarose beads. Samples were resolved on a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis under reducing conditions. A VV wild-type-infected sample (wt) was used as a negative control.
induced SAg responses similar to v-GR. These results demonstrate that all rVVVs produce parental/hybrid molecules with intact SAg function that, except for v-7G1, induce quantitatively similar SAg responses in I-E\(^{+}\)/H11001 mice (200 to 250% of normal control). Thus, all hybrid SAgs adopt a three-dimensional conformation that allows correct transport to the cell surface, association and presentation with I-E, and recognition by the specific T-cell receptor V\(\beta\)14 chains as the two parental SAgs.

In I-E\(^{-}\) mice, v-GR induced a barely detectable increase of specific V\(\beta\)14\(^{+}\) and nonspecific V\(\beta\)6\(^{+}\) CD4\(^{+}\) lymphoblasts of uninjected control mice. Means ± standard errors were also indicated for each group. In I-E\(^{-}\) mice, only v-7G3 was able to efficiently stimulate V\(\beta\)14\(^{+}\) CD4\(^{+}\) T cells, whereas v-7G4 and v-7G5 gave SAg responses similar to that of v-GR (Fig. 3B, D). Based on the comparison with GR of their ability to stimulate CD4\(^{+}\) T cells in the absence of I-E, irrespective of their V\(\beta\) specificity, our hybrid SAgs can be divided into two groups: hybrids 7G1, 7G2, 7G3, and 7G6 behave like I-E-independent SAgs and can be presented by MHC class II I-A, whereas hybrids 7G4 and 7G5 behave as I-E-dependent SAgs.

Our results demonstrate a dominant contribution of the C-terminal third of mtv-7 SAg in the association with I-A in an in vivo setting (7G2). This reveals a major site of interaction with I-A in the C-terminal 111 aa (aa 212 to 322) or more precisely, in the C-terminal 90 aa, due to the lack of amino acid difference between GR and mtv-7 (Fig. 1). Previous biochemical analyses have indeed proposed a binding site for MHC class II HLA-DR, the human homologue of I-E, in the C-terminal 18.5-kDa fragment of mtv-7 (10). Here, we further show that separately, the membrane-proximal (7G4) and the

![Graph A](https://via.placeholder.com/150)

**FIG. 3.** SAg responses induced by rVV expressing hybrid mtv-7/GR SAgs in I-E\(^{+}\) and I-E\(^{-}\) mice. I-E\(^{+}\) mice (BALB/c or C57BL/6 I-E\(^{+}\)tg [7]) and I-E\(^{-}\) mice (C57BL/6) (Harlan OLAC Ltd., Bicester, United Kingdom) were injected subcutaneously in the hind footpads with rVV (10\(^{6}\) PFU in a 30-\(\mu\)l volume). Twenty hours postinjection the mice were sacrificed, and the draining popliteal lymph nodes were isolated and homogenized to a single-cell suspension. Cells were stained with a mixture of the given anti-V\(\beta\) antibody (anti-V\(\beta\)6FITC [44.22.1]) (1) or anti-V\(\beta\)14FITC (14-2; BD Pharmingen, San Diego, Calif.) and anti-CD4PE antibody (RM4-5; BD Pharmingen) in a single step. Analysis was performed on a FACScan (Becton Dickinson, Mountain View, Calif.) cell analyzer by using the Lysis II software for data evaluation. Dead cells were excluded on the basis of their forward and side scatter characteristics. Twenty thousand to 50,000 cells were acquired. Since responses in C57BL/6 mice were in general low, each rVV expressing a hybrid SAg was tested in at least four experiments. Cumulative results of all performed experiments are expressed as the percentage of V\(\beta\)14 CD4\(^{+}\) lymphoblasts of uninjected control mice. Means ± standard errors were also indicated for each group. In I-E\(^{-}\) mice, v-7, v-7G1, v-7G2, v-7G3, and v-7G6 induced responses that are significantly different from those of v-GR (analysis of variance; Dunnett’s post test [\(P < 0.05\)]), whereas v-7G4 and v-7G5 gave responses similar to that of v-GR (\(P > 0.05\)).
middle (7G5) thirds of the extracellular domain of the \textit{mtv-7} SAg do not support interaction with I-A, but they do so when combined (7G3). This suggests either that both the membrane-proximal and middle thirds of the SAg contain structures that cooperate for association with I-A and/or that one site mediating I-A interaction overlaps the junction of the two thirds around aa 122. Previous studies by others addressed MHC class II binding sites in the membrane-proximal third. Using competition assays for peptide binding, aa 76 to 119 of the \textit{mtv-1} SAg were identified as a common binding site for I-E and I-A, even though the function of \textit{mtv-1} competition assays for peptide binding, aa 76 to 119 of the \textit{mtv-1} SAg and I-A, even though the function of \textit{mtv-1} SAg is I-E dependent in vivo (11). Mutagenesis analyses showed, however, that amino acids surrounding residue K109 were not involved in the interaction with MHC class II (9). Another recent report further suggested that aa 86 to 94 are necessary but not sufficient for the association of MMTV SAg with I-E, in a manner similar to that of the invariant chain (4). Based on the lack of correlation between I-E dependence and amino acid variability at SAg residues 86 to 94 and on the observation that all our wild-type and hybrid SAg show intact SAg function, when presented by I-E, we propose that all I-E-dependent and -independent viral SAg can interact with I-E and I-A via residues 86 to 94. Since this interaction is not sufficient, other binding sites with differential affinities for I-E versus I-A, thereby determining the I-E dependence or independence of a SAg, are required for stable association with I-E or I-A, as shown in this paper. Although in full agreement with the studies of Hsu et al. (4) and Torres et al. (11), our approach does not address common binding sites for I-E and I-A, such as aa 86 to 94.

In conclusion, our results are compatible with the idea that different binding sites on the \textit{mtv-7} SAg cooperate for efficient association with MHC class II I-A (4). Alternatively, we cannot exclude the possibility that nonlinear structures in the GR SAg impede I-A but not I-E binding directly or indirectly by changing the overall three-dimensional conformation of the molecule.

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