Structural Analysis of a Mouse Mammary Tumor Virus Superantigen

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Summary

It has recently been shown that the minor lymphocyte stimulating–like products expressed by some mice are actually encoded by open reading frames in the 3' long terminal repeats of mouse mammary tumor viruses. These products act as viral superantigens (vSAGs). That is, they stimulate most T cells bearing particular Vβs almost regardless of the rest of the variable components of the T cell receptors expressed by those cells. To find out more about the structure of these vSAGs, a set of truncated vSAG genes was used in transfection and in vitro translation experiments to show that the functional vSAG is a type II integral membrane protein with a large glycosylated extracellular COOH-terminal domain and a small, nonessential, intracellular NH$_2$-terminal cytoplasmic domain. These results are consistent with the fact that the vSAGs must be expressed on the cell surface in order to interact with T cells and class II major histocompatibility complex proteins. They also account for the finding that much of the Vβ specificity of the vSAGs is controlled by amino acids at the COOH-terminal end of the vSAG proteins, amino acids that will be extracellular in type II proteins.

It is almost 20 years since Festenstein (1) discovered the Mls loci in mice. At the time he identified their products by their ability to stimulate very powerful mixed lymphocyte reactions between spleen cells of mice identical at their MHC loci, but different in background genes. Since the discovery of Mls, it has been shown that the products of these genes, in conjunction with class II MHC proteins, stimulate Mls-disparate T cells (2, 3). Also, it is now known that Mls products are superantigens, antigens that bind to MHC and stimulate virtually all T cells bearing particular Vβ regions as part of their receptors (4, 5). It is this property that makes some of the Mls products such powerful T cell stimulants. Mice have only about 20 different Vβ regions available for use as part of their receptors and therefore any given Mls product will stimulate 5% or more of the T cells in a mouse of appropriate MHC type.

Although the phenomena of Mls have been known for some time, it was not until recently that the genes coding for Mls products were identified. Various Mls-like products have now been shown to map concordantly with mouse mammary tumor virus (MTV)[1] integrants in the genomes of certain strains of mice (6–10). In the case of two exogenous MTVs, we and others have shown that their associated viral superan-

tigens (vSAGs) are encoded by the open reading frames (ORFs) in the 3' LTRs of the viruses (10–12). The exact structure of the product of the vSAG gene was, however, heretofore unknown.

To find out more about the actual structure of the vSAg protein, we constructed full-length and truncated versions of the gene encoded by the C3H/HeJ exogenous MTV (exoMTV) and studied the properties of their protein products. We report here that the exoMTV vSAG is a type II membrane-bound glycoprotein, with a core protein of 37 kD (the predicted molecular mass of the full-length ORF gene protein product), and that most of the short NH$_2$-terminal cytoplasmic tail of this protein is not essential for its function.

Materials and Methods

Construction of ORF Wild-Type and Truncated Genes. The synthesis of the exoMTV full-length ORF gene was described previously (11). Briefly, this coding region (ORFml) was amplified from a fragment of MTV containing env and the 3' LTR (a gift of Dr. J. Majors, Washington University, St. Louis, MO) using a PCR (13) and MTV-specific 5' and 3' oligonucleotides. Truncated versions of the gene, ORFm2-m5, were produced in similar fashion. The sequences of the 5' primers for these constructs were as follows: 5'-GGGAATTCTCGAGATGCCGCGCCTGCAG-3', ORFml; 5'-GGGAATTCCGGGATGAGCCCATCAGACAAAG-3', ORFm2;

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[1] *Abbreviations used in this paper:* exoMTV, exogenous mouse mammary tumor virus; ORF, open reading frame; vSAG, viral superantigen.

5'-GAATGCTTGCTAAAAATTAT-3', ORFm3; 5'GGGAATTCCA-CTATGTTAAGAAATGAATCATTA-3', ORFm4; 5'-GGGAATTC-CGAAATGGGAATAGAAAATAGA-3', ORFm5. The 3' primer for all the ORF genes was 5'-GGGGATCCTCTAGAGGGAACCG-CAAGGTTGGG-3'. The amplified products were cloned into pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ), sequenced, and ligated into the mammalian expression vector pBDWMCβ2, as described previously (11, 14).

Transfection of ORF Gene Products. The full-length and truncated ORF genes, ORFm1-m5, were transfected into the B cell hybridoma, LBB.1 (11, 15). Transfectants were selected by growth in G418 and were screened for expression of TCR Cβ mRNA, encoded by pBDWMCβ2, by Northern blot analysis using a mouse Cβ probe.

In Vitro Protein Synthesis. The ORFm1-3 sequences carried in pTZ18R plasmids were used to synthesize capped mRNAs in vitro with T7 RNA polymerase using the in vitro transcription kit as suggested by the supplier (Stratagene, La Jolla, CA). 1 μg of capped mRNA was subjected to in vitro translation with a rabbit reticulocyte lysate preparation (Promega Biotec, Madison, WI). In some cases canine microsomal membranes (Promega Biotec) were added to the translation cultures. Synthesized protein was labeled by the addition of ^{35}S-methionine and ^{35}S-cysteine to the reaction mixtures. Products were analyzed on 10% SDS-polyacrylamide gels. To demonstrate how much of the ORF proteins was transported into microsomes, in some cases translation products obtained in the presence of microsomal membranes were incubated for 10 min on ice with 50 μg/ml proteinase K with or without 0.1% Triton X-100 before analysis on SDS-polyacrylamide gels.

Assay of ORF Function. LBB.1 transfectants were screened for their ability to stimulate the T cell hybridomas K14-15.11 (Vβ14⁺) and KOX15-8.3 (Vβ15⁺) because the vSAg encoded by exoMTV is known to stimulate mouse T cells bearing either of these Vβs (11). The T cell hybridomas DO-11.10/S4.4 (Vβ8.2⁺) and KOX7-6.6 (Vβ7⁺) were used as positive and negative controls, respectively. T cells bearing Vβ8.2 are not stimulated by any of the vSAgs expressed endogenously by LBB.1. T cells bearing Vβ7 are stimulated by an LBB.1 endogenous vSAg (11). Stimulation of the hybridomas was assayed by lymphokine production as previously described (16).

Results

Which Methionine Is the Start Site for Active ORF Proteins? The predicted protein sequence of the ORF gene from C3H/HeJ exoMTV is shown in Fig. 1 (17, 18). The sequence contains more than five methionine residues. Since the product of the ORF gene has never been detected in vivo, the actual translation initiation site was unknown. The longest protein that might be encoded by the ORF gene would be 37 kD, but other potential start sites would encode proteins of ~33, ~23, ~20, and ~18 kD.

To find out which methionine was actually used as the start site by the exoMTV ORF product, four NH₂-terminally truncated ORF genes were created that would allow initiation of protein synthesis at successive methionines within the molecule (ORF sm2-5). These genes, and a control full-length exoMTV ORF gene (ORFm1), were cloned into the mammalian expression vector, pBDWMCβ2, and transfected into MHC class II-expressing LBB.1 B hybridoma cells. Transfectants were assayed for stimulation of T cell hybridomas
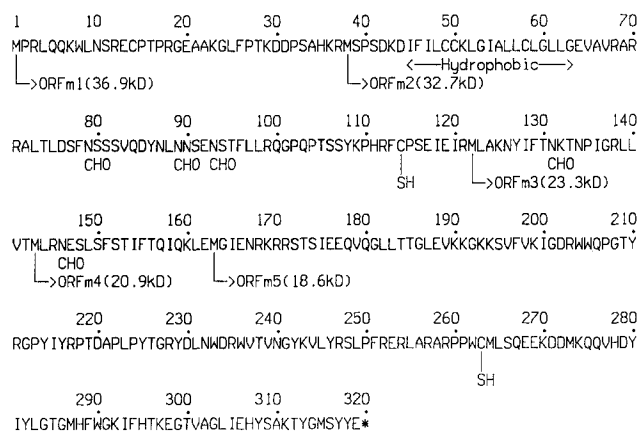


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1      10        20        30        40        50        60        70
MPRLQQKWLNSRECPTPRGEAAKGLFPTKDDPSAHKRMSPSDKD IF ILCCKLG IALLCLGLLGEVAVRAR
                                               <——Hydrophobic——>
 └─>ORFm1(36.9kD)                          └─>ORFm2(32.7kD)

      80        90        100       110       120       130       140
RALTLDSFNSSSVQDYNLNNSENSTFLLRQGPQPTSSYKPHRFCPSE IE IRMLAKNY IFTNKTNP IGRLL
    CHO          CHO CHO                       |          CHO
                                              SH    └─>ORFm3(23.3kD)

     150       160       170       180       190       200       210
VTMLRNESLSFST IFTQ IQKLEMG IENRKRRSTS IEEQVQGLLTTGLEVKKGKKSVFVK IGDRWWQPGTY
    CHO
 └─>ORFm4(20.9kD)    └─>ORFm5(18.6kD)

     220       230       240       250       260       270       280
RGPY IYRPTDAPLPYTGRYDLNWDRWVTVNGYKVLYRSLPFRERLARARPPWCMLSQEEKDDMKQQVHDY
                                                         SH

     290       300       310       320
IYLGTGMHFWGK IFHTKEGTVAGL IEHYSAKTYGMSYYE*
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Figure 1. The predicted amino acid sequence of exoMTV ORF. The sequence is deduced from the DNA sequence reported for this gene in references 17 and 18. Potential sites for N-linked glycosylation are indicated by asterisks. A potential signal peptide/transmembrane hydrophobic region is also marked. The expected sizes of the protein encoded by the full-length and truncated ORF genes are shown in parentheses beside the names of each gene.

expressing Vβ14 and Vβ15, the targets of the exoMTV vSAG (11). The results of a representative experiment are illustrated in Fig. 2. As previously shown (11), the untransfected cell line, LBB.1, failed to stimulate K14-15.11 (Vβ14) and KOX15-
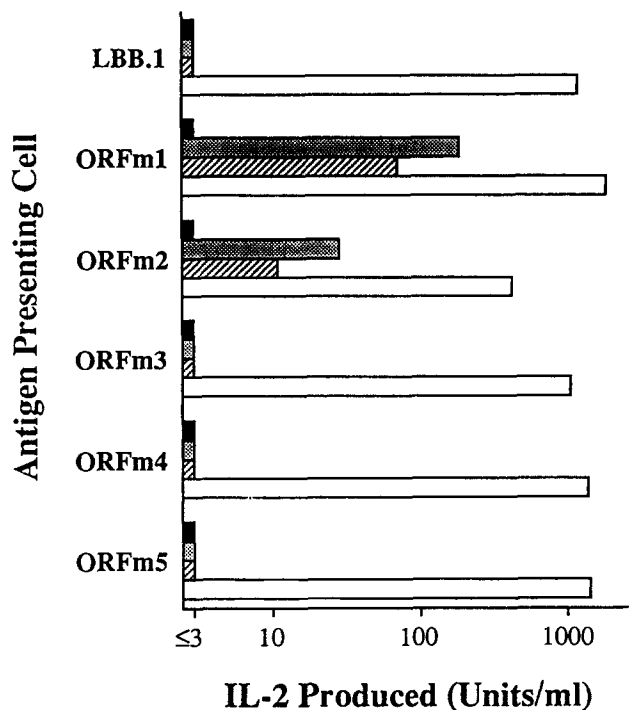

IL-2 Produced (Units/ml)

Figure 2. The effect of NH₂-terminal truncation on the superantigen activity of exoMTV ORF. LBB.1 B lymphoma cells transfected with full-length or truncated MTV ORF genes were tested for their ability to stimulate various T cell hybridomas as previously described (16). Stimulation was assayed 24 h later by levels of secreted lymphokines in the supernatants. (▨) Response of K14-15.11 (Vβ14⁺); (▩) response of KOX15-8.3 (Vβ15⁺); (■) response of DO-11.10 (Vβ8.2⁺); (□) response of KOX7-6.6 (Vβ7⁺).
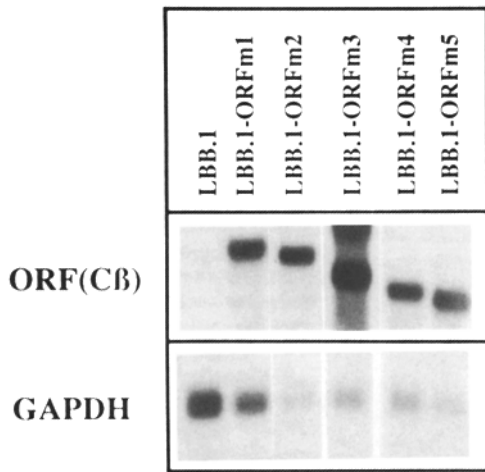
Figure 3. Northern blot analysis of B cell transfectants. Poly(A)$^+$ RNA was purified from untransfected LBB.1 and the ORF transfected derivatives, and analyzed by Northern blotting as previously described. Messenger RNA from the transfected ORF constructs was detected using a mouse TCR Cβ probe, which detects the 3' untranslated region of the construct mRNA (11). The amount of RNA loaded in each lane was normalized by the amount of RNA hybridized with a probe for glyceraldehyde phosphate dehydrogenase (GAPDH).

8.3 (Vβ15). Transfectants expressing ORFs truncated to the third methionine in the predicted protein and beyond (LBB.1-ORFm3-5) were also inactive in this assay. Transfectants carrying the full-length ORF gene, or a truncated ORF gene that could initiate at the second methionine (LBB.1-ORFm1 and LBB.1-ORFm2, respectively), were active in this assay. LBB.1-ORFm2 was less efficient than LBB.1-ORFm1, however. A Vβ8.2$^+$ T cell hybridoma, DO-11.10/S4.4, was not stimulated by any of the B cell lines, as expected. All the B cell lines had the ability to present MTV vSAGs expressed within them to T cells, since all the B cell lines stimulated a Vβ7-bearing T cell hybridoma, KOX7-6.6, by means of a Vβ7-specific vSAG endogenous to LBB.1 (11; Dr. L. Ignatowicz, personal communication).

To check that all these cell lines expressed the transfected ORF genes at comparable levels, mRNA was prepared from the different transfectants and analyzed by Northern blot for expression of mouse Cβ mRNA. In pBDWMCβ2 constructs, the ORF genes and variants are encoded upstream of the mouse Cβ2 gene, which will be cotranscribed with the ORF genes themselves. Expression of Cβ2 mRNA in these transfectants should, therefore, accurately reflect transcription of the various ORF constructs. The LBB.1 transfectants could not be analyzed for expression of the transfected ORF genes themselves because LBB.1 expresses several endogenous ORF genes that would crosshybridize extensively with the transfected gene products. Transfected mRNA was also analyzed for levels of GAPDH mRNA, which is mRNA for a housekeeping gene.

As shown in Fig. 3, LBB.1-ORFm1 expresses less Cβ mRNA by comparison with GAPDH mRNA than the other transfectants. The other transfectants express roughly equivalent levels of Cβ mRNA. These data show that the results

described above are not the consequence of low ORF mRNA expression in the inactive transfectants. They also suggest that the difference in activity between LBB.1-m1 and LBB.1-m2 is not due to lower levels of ORF expression in the latter cell line, but rather may be due to stronger activity on the part of the full-length ORF protein.

These results establish several points. First, they show that the ORF protein initiating from either the first or second methionine residue can function as a vSAG with proper Vβ specificity. Second, internal methionine residues COOH terminal to the second methionine cannot initiate an ORF gene product with superantigenic properties. Finally, the first 38 amino acids of the predicted protein sequence of full-length exoMTV ORF are not essential for the function of the protein, although they may contribute somewhat to the efficiency with which it acts.

Can the ORF Protein Be Transported into Microsomes and Glycosylated? If the ORF protein is to be recognized by T cells it must find its way to the cell surface. The predicted product of the ORF gene does have a single hydrophobic region (Fig. 1), which could act as a signal peptide and/or transmembrane region. If the protein is transported into the microsomal compartment it should be glycosylated. The predicted sequence for the ORF product does have five potential sites for N-linked glycosylation, all, interestingly enough, COOH terminal of the hydrophobic stretch of amino acids (Fig. 1). To find out whether or not the protein could be glycosylated, and, if so, to determine the function of the hydrophobic region in this process, we used in vitro translation systems to synthesize the protein.

The ORFm1, ORFm2, and ORFm3 genes described above were used as substrates for mRNA synthesis. Equal amounts of mRNA were translated in vitro using a rabbit reticulocyte lysate system, and analyzed by SDS-PAGE. Each transcript produced proteins of the expected size (Fig. 4 *A, left*). That is, ORFm1, ORFm2, and ORFm3 transcripts yielded proteins of 37, 33, and 23 kD, respectively, although in each case some initiation of translation was seen from subsequent internal methionines as well. All the transcripts were translated in vitro with roughly the same efficiency.

To study the cotranslational processing or modification of these proteins, the same mRNAs were translated in vitro in the presence of microsomal membranes (Fig. 4 *A, right*). When either the ORFm1 or ORFm2 transcripts were translated in the presence of microsomal membranes, four additional bands with higher molecular masses than the core proteins of 37 and 33 kD appeared, consistent with glycosylation of the core proteins at four of the five potential N-linked glycosylation sites contained in these proteins (Fig. 1). Perhaps steric hindrance prevents glycosylation on the same protein molecule at both the nearly adjacent sites at residues 89 and 93.

For both ORFm1 and ORFm2 products, the glycosylated forms of the proteins lay on a ladder with intervals of ~3 kD, the expected molecular mass of an immature N-linked sugar complex. Despite the presence of two potential N-linked glycosylation sites in the mORF3 product, there was no evidence for its glycosylation in the presence of microsomes.
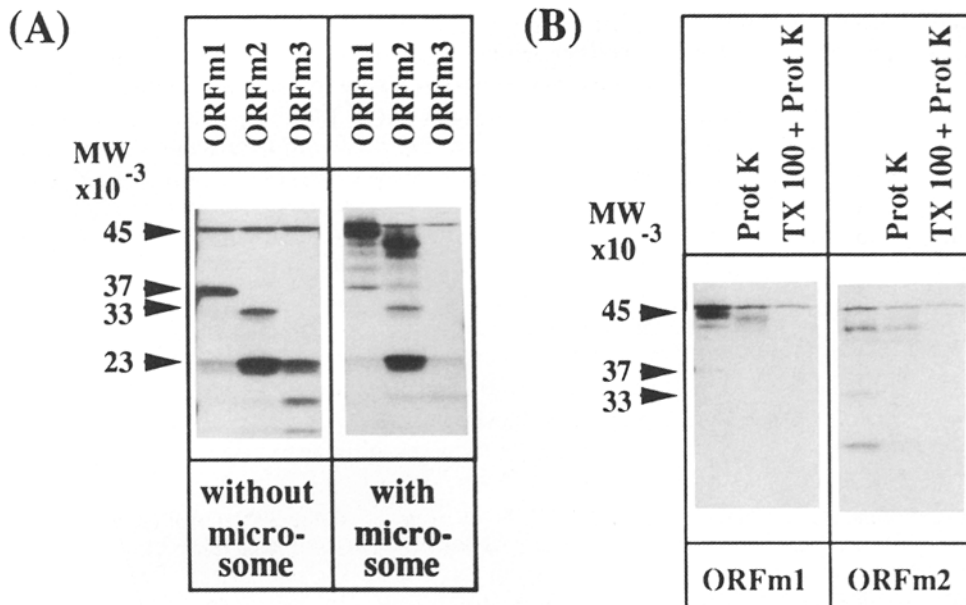
These results showed that the ORFm1 or ORFm2, but

Figure 4. The ORF gene product is a type II membrane spanning glycoprotein. (A) ORFm1, ORFm2, and ORFm3 sequences carried in the plasmid pTZ18R (Pharmacia) were used to synthesize capped mRNAs in vitro. 1 μg of capped mRNA was subjected to in vitro translation with a rabbit reticulocyte lysate preparation without (left) or with (right) canine microsomal membranes (Promega Biotec). Synthesized protein was labeled by addition of ^{35}S-methionine and ^{35}S-cysteine to the reaction mixture. Products were analyzed on 10% SDS-polyacrylamide gels. The band at 45 kD was present in all samples and is produced from mRNA contaminating the transcription kit. (B) The in vitro translation products from ORFm1 (left) or ORFm2 (right) prepared with canine microsomal membranes as in A were incubated with proteinase K (50 μg/ml) for 10 min on ice with or without Triton X-100 (0.1%) and analyzed on SDS-PAGE.

not the ORFm3, gene products can be transported into the endoplasmic reticulum such that sites COOH terminal to the hydrophobic region can be N-glycosylated. Furthermore, the molecular masses of the translation products do not suggest any truncation of the protein in the endoplasmic reticulum. This result strongly suggests that the hydrophobic region beginning at amino acid 45 of the full-length ORF protein acts as a signal peptide that is not cleaved after transport into the endoplasmic reticulum.

Is the ORF Protein an Integral Membrane Protein? To confirm this result and determine whether the ORF product is an integral membrane protein, microsomal membranes containing ORFm1 or ORFm2 proteins were treated with proteinase K, and the protected protein fragments were analyzed by SDS-PAGE (Fig. 4 B). Most of the glycosylated ORFm1 protein (left) and all of the ORFm2 protein (right) was protected from proteinase K treatment unless the microsomes were disrupted by detergent. This confirmed that the vast majority of the protein ended up inside the endoplasmic reticulum. However, a small portion (∼2 kD) of the ORFm1, but not ORFm2, product was removed by proteinase K treatment, indicating that the small NH2-terminal portion present in the ORFm1 product, but absent from ORFm2, carries a protease-sensitive site and remains outside the microsomes. This indicates that ORF is indeed a membrane protein with a small cytosolic NH2-terminal portion and a transmembrane region, with the majority being a COOH-terminal end in the extracellular compartment.

Discussion

Although Mls antigens, the MTV vSAGs, are recognized like conventional antigenic peptides in association with MHC by T cells, the recent discovery that these products are superantigens (4, 5) suggested that they may not act as peptides but rather as proteins of appreciable size, comparable with that of the other well-characterized superantigens, the bacterial toxins (19–21).

The data in this paper strongly support this latter idea. The gene truncation experiments show that in order for the vSAG to engage T cells, it must contain the hydrophobic region of amino acids near the NH2-terminal end of the protein. The in vitro translation experiments described here show that this hydrophobic stretch of amino acids can act both as a signal peptide for transport into the endoplasmic reticulum and as a transmembrane region.

The experiments described here also show, perhaps surprisingly, that the MTV SAGs are type II membrane-bound proteins. Most membrane proteins are type I, that is their NH2-terminal amino acids are found outside and their COOH-terminal amino acids lie inside the cell. Type II membrane proteins, on the other hand, have a single internal hydrophobic region, which acts both as a signal peptide and as a transmembrane region, such that the COOH terminus of the protein is found extracellularly, and the NH2 terminus is found in the cytoplasm (22–25). Our finding that most of the vSAG protein is protected from proteases after transport into microsomes, and that only a small NH2-terminal fragment can be digested in such experiments, shows that the vSAGs are likewise type II integral membrane proteins. This conclusion is consistent with the idea that the extremely variable COOH-terminal residues of this group of proteins may contribute to their Vβ specificity (11, 17, 18, 26–29). It is also consistent with our finding that the NH2-terminal cytoplasmic region is not absolutely required for vSAG function, but may improve its efficiency. Also, the anchoring of

vSAGs in the plasma membrane should enhance their ability to interact with class II MHC molecules, a prerequisite for T cell recognition.

The hydrophobic putative signal peptide/transmembrane region of this protein is flanked by a negatively charged aspartic acid immediately on the NH₂-terminal side, a feature not characteristic of most type II proteins, which usually have a positively charged amino acid at this position (23-25). An-

other unusual property of the hydrophobic region is an internal positively charged lysine, of unknown functional significance, a feature shared with the transmembrane regions of the α and β chains of the TCR (30, 31), where the function appears to be to promote association with CD3 subunits that bear negatively charged amino acids in their transmembrane regions.

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