The Mouse Mammary Tumor Virus Envelope Gene Product Is Required for Superantigen Presentation to T Cells

By T. V. Golovkina,* 1 A. Chervonsky,† J. A. Prescott,* C. A. Janeway, Jr.,‡ and S. R. Ross*

From the *Department of Biochemistry, University of Illinois College of Medicine, Chicago, Illinois 60612; and the †Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06510

Summary

Transgenic mice expressing either the mouse mammary tumor virus (MMTV) superantigen gene (sag) alone or in combination with the viral envelope genes (env) (LEL), or all of the viral genes (gag, pol, env, and sag) (HYB PRO), deleted VB14+ T cells from their immune repertoire. However, only LEL or HYB PRO transgenic antigen-presenting cells were capable of stimulating a proliferative response from nontransgenic primary T cells or interleukin 2 production from a VB15-bearing T cell hybridoma. These T cell responses could be inhibited by a monospecific antibody directed against the MMTV gp52 cell surface glycoprotein. These results indicate that the MMTV gp52 gene product participates in the presentation of superantigen to T cells, resulting in their stimulation, a requisite step in the MMTV infection pathway. Thus, gp52 could play a role in the transfer of virus between different subsets of lymphocytes.

Mouse mammary tumor virus (MMTV)2 is a nonacute transforming retrovirus that induces mammary adenocarcinomas in susceptible mice after a relatively long latency period (1). MMTV is transmitted by two routes: genetically as an endogenous provirus and exogenously as milk-borne infectious virus particles. Endogenous MMTV proviruses have been found in all commonly used strains of laboratory mice; however, the majority of these do not produce functional viral particles capable of being transmitted exogenously (2).

The MMTV protein products are translated from three viral transcripts (3). Two polyproteins are translated from a genomic-length RNA (Fig. 1), the 77-kD polyprotein that is cleaved to form the viral core proteins and the 180-kD polyprotein Pr180g~r~l reverse transcriptase precursor. The 70-kD polyprotein that is processed to form the gp52 and gp36 envelope (Env) glycoproteins encoded in the 3′ half of the MMTV genome comes from an internally spliced transcript. Because MMTV viral particles are produced by budding, infected cells express gp52 and gp36 on their cell membrane (4). Gp36 is the transmembrane domain of Env, while the cell surface gp52 Env protein on viral particles binds the cellular receptor for MMTV (5). Finally, a fourth protein product, the viral superantigen (Sag), is synthesized from a spliced RNA encoded primarily in the two long terminal repeats (LTRs) of the provirus. This protein acts as an Mls antigen (for review, see references 6 and 7) and plays an important role in the viral life cycle (8, 9).

We recently generated C3H/HeN inbred transgenic mice that express the C3H exogenous virus sag gene under the control of the MMTV LTR (Fig. 1) (termed MTV-open reading frame [ORF] transgenic mice) (8). Expression of this sag gene resulted in the deletion of cognate VB14-bearing T cells from the immune repertoire of MTV-ORF transgenic mice and also protected these animals from being infected by milk-borne exogenous C3H virus. As a consequence, they had a much lower incidence and greater latency of MMTV-induced tumors (10, 11).

We show here that although the expression of the sag transgene caused deletion of cognate T cells in MTV-ORF mice, spleen cells from these animals were unable to stimulate an Mls response in vitro. In contrast, splenocytes from transgenic mice that contained either an entire provirus, including the LTRs, sag, pol, and env genes (hybrid provirus [HYB PRO]) or a deleted provirus that had only the LTRs and env genes (LTR/env/LTR [LEL]) stimulated proliferation of primary spleen cells and IL-2 production by a VB15-bearing T

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1 T. V. Golovkina and A. Chervonsky made equal contributions to this paper.

2 Abbreviations used in this paper: HYB PRO, hybrid provirus; LEL, LTR/env/LTR; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; MTV-ORF, mammary tumor virus-open reading frame; NGG, γ-globulin fraction of normal goat serum.
MATERIALS AND METHODS

Plasmids. The plasmid hybrid MMTV used to make the HYB PRO transgenic strain was a gift from G. M. Shackleford, University of California, Los Angeles (12) (Fig. 1). It contained the 5' half of the Mtv-1 endogenous provirus (to the EcoRI site in pol) and the 3' half of an integrated copy of C3H exogenous virus (Fig. 1). The LEL plasmid was constructed by cloning a HindIII-HindIII fragment from the hybrid MMTV (containing the env gene and 3' LTR) downstream of a phasmid carrying a LTR from exogenous Mtv loci present in the C3H/HeN genome. Because there are sequence dissimilarities in the 3' end of viral transcripts derived from the endogenous and exogenous viruses (the 3' LTR of all the transgenes used came from exogenous C3H MMTV (Fig. 1)), a Sau3A-Sau3A (-738 bp to -110 bp) fragment from exogenous C3H MMTV was cloned into the BamHI site of pBluescript II SK vector (Stratagene, La Jolla, CA) and used to create a probe. Labeled RNA probes were synthesized from the vector template using [α-32P]UTP (ICN Biomedical Inc., Irvine, CA) and T3 RNA polymerase as specified by the supplier (Promega Biotech, Madison, WI). An excess of labeled probe (5 × 10^6 cpm/reaction) was hybridized to 40 μg of total RNA. Hybridizations were performed overnight at 56°C in 30 μl of hybridization buffer (40 mM piperazine-N,N'-bis[2-ethanesulfonic acid], pH 6.4, 0.4 M NaCl, 80% formamide). Approximately 500 U of RNase T1 (GIBCO BRL, Gaithersburg, MD) was added to each reaction in 2 ml of complete media. After 4 d of culture, the surviving T cells were separated from dead cells on Ficoll/Hypaque density gradients, stained with anti-CD4 and anti-Vβ14 or anti-Vβ6 antibodies, and analyzed by FACS®.

IL-2 Production Assays. Stimulator spleen cells (10^5) and 5 × 10^4 VS15-positive Kox 15-8.3 hybridoma cells (18) in 200 μl of complete media were cocultured overnight in triplicate. IL-2 production was measured by [3H]thymidine (ICN Biomedicals, Inc.) incorporation into the IL-2-dependent cell line CTLL-2.

Western Blot Analysis and Antibody-blocking Studies. MMTV virions were purified from C3H/HeN MMTV^+ milk as described (19). Viral antigens that were recognized by the polyclonal goat anti-MMTV serum as well as by the monospecific goat affinity-purified anti-gp52 antibody (Quality Biotech Inc., Resource Laboratory, Camden, NJ) were detected by Western blot analysis. Briefly, 4 μg of purified MMTV virus particles was electrophoresed on 10% SDS-polyacrylamide gels and electrotransferred to nitrocellulose. The nitrocellulose strips were incubated with either anti-MMTV (140 μg/ml) or with anti-gp52 antisera (2 μg/ml) followed by rabbit anti-goat antiserum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The rabbit antibodies were detected with 125I-labeled protein A (ICN Biomedical Inc.).

For the antibody-blocking studies, the anti-gp52, anti-gp36, and anti-p27 antibodies (Quality Biotech Inc., Resource Laboratory), as well as γ-globulin fraction of normal goat serum (NGG) (Jackson ImmunoResearch Laboratories, Inc.) were dialyzed against 4,000 vol of PBS overnight at 4°C, diluted in complete media at a concentration of 80 μg/ml, and filter sterilized. MLC assays were performed in triplicate as described above in the presence of anti-gp52 antibodies or NGG at the final concentrations indicated in Fig. 6. The percent inhibition was calculated as follows: percent inhibition = [(a - b)/a] × 100, where a represents the incorporation in the presence of NGG and b represents the incorporation in the presence of the same concentration of anti-gp52 antibody.

For the pseudosorption of the anti-gp52 antibody, ~10^5 splenocytes from nontransgenic C3H/HeN or transgenic HYB PRO mice per microgram of antibody were incubated for 1 h at room temperature, followed by 1 h at 4°C. The cells were pelleted and the supernatant used in blocking experiments as described above.

RESULTS AND DISCUSSION

In addition to the MTV-ORF transgene previously described (8), we used two constructs to create transgenic mice in the C3H/HeN inbred background. One strain, termed HYB PRO (12), contained as a transgene an entire copy of a genetically engineered MMTV provirus in which the 3' half (including 3' LTR, env, and gag genes) was derived from C3H exogenous virus and the 5' half (including 5' LTR, pol, gag, and pol genes) came from the Mtv-1 endogenous locus already present in C3H/HeN mice (Fig. 1). The second con-
Figure 1. Diagram of the HYB PRO, LEL, and MTV-ORF transgenes. (Top) HYB PRO transgene and the three transcripts generated from this provirus, including the viral protein coding regions. The LEL construct was cloned as described in Materials and Methods; only one RNA coding for both the env and sag genes can be transcribed. (Bottom) MTV-ORF transgene and transcript. The filled box represents the region of the LTR used as a probe for RNase T1 protection analysis. H, HindIII; R, EcoRI; S, Sau3A.

struct used to microinject embryos, LEL, had a deletion of all of the gag and most of the pol coding regions, but retained the env gene as well as the 5' and 3' LTRs from C3H exogenous virus (Fig. 1). One strain of HYB PRO mice and two independently derived strains of LEL mice were used in these strains.

The presence of a self sag gene or Mls locus in mice is characterized by the absence of mature T cells bearing receptors encoded by a particular Vβ gene segment (20–23). To determine whether the HYB PRO and LEL transgenic mice produced functional Sag protein, the percentage of Vβ14+ T lymphocytes present in their periphery was measured. Similar to what was seen with MTV-ORF transgenic mice, the HYB PRO as well as LEL mice showed specific deletion of both CD4+Vβ14+ and CD8+Vβ14+ T cells (Table 1). Thus, all three types of transgenic mice, MTV-ORF, LEL, and HYB PRO, demonstrated one of the characteristics of Mls (in other words, they deleted cognate T cells).

The Mls genes were originally named for their ability to stimulate the proliferation of specific Vβ-bearing T cells in MLC assays of spleen cells isolated from mouse strains identical at the MHC (24). We therefore determined if primary spleen cells from these different transgenic mice could present the Sag protein in in vitro MLC assays. Primary spleen cells from MTV-ORF, LEL, and HYB PRO transgenic mice were tested for their ability to stimulate the proliferation of C3H/HeN MMTV nontransgenic primary lymph node cells or splenocytes. No splenocytes isolated from any of the three different strains of MTV-ORF transgenic mice tested (nos. 13, 16, and 35; reference 8) could stimulate proliferation of the nontransgenic responder cells (Fig. 2 A). Moreover, even after activation with LPS, the MTV-ORF splenocytes did not stimulate the responder cells (not shown). In contrast, spleen cells from both the HYB PRO and LEL transgenic mice caused proliferation of these same responder cells (Fig. 2 A), and LPS blasts prepared from these mice caused even higher stimulation than primary splenocytes (not shown). The HYB PRO splenocytes stimulated the responder cells ~2-fold more than those isolated from either strain of LEL mice, which was probably a reflection of the higher level of transgene expression (see below).

To ensure that the proliferative response was directed against the Sag protein encoded in the LTR, we first tested whether the Vβ15-bearing hybridoma cell line Kox 15-8.3 (18), which recognizes the C3H MMTV Sag, responded to the transgenic APCs. We have previously shown that only hybridomas bearing Vβ14 or Vβ15 TCRs were stimulated by HYB PRO APCs (10). The Kox 15-8.3 cells produced IL-2 when stimulated with primary splenocytes from both the HYB PRO and LEL, but not MTV-ORF transgenic mice (Fig. 3), showing that the Sag protein was presented to cognate T cells bearing Vβ14+.

Table 1. Deletion of Vβ14+ T Cells in MTV-ORF, HYB PRO, and LEL Transgenic Mice

| Mice              | Transgene RNA level | CD4/Vβ14 | CD8/Vβ14 |
|-------------------|---------------------|-----------|-----------|
| C3H/HeN MTV      | N.A.                | 8.2 ± 1.1 | 9.5 ± 1.3 |
| MTV-ORF 13       | High                | 2.1 ± 0.2 | 2.3 ± 0.4 |
| MTV-ORF 16       | High                | 1.2 ± 0.2 | 2.4 ± 0.4 |
| MTV-ORF 35       | High                | 1.8 ± 0.4 | 2.3 ± 0.2 |
| HYB PRO          | High                | 0.8 ± 0.4 | 1.6 ± 0.8 |
| LEL 1            | Low                 | 1.5 ± 0.2 | 3.2 ± 0.2 |
| LEL 2            | Low                 | 2.0 ± 0.5 | 3.3 ± 0.2 |

* T cells were isolated from the peripheral blood or lymph nodes of the mice indicated and analyzed for the percentage of CD4- or CD8-bearing Vβ14+ T cells. The numbers shown represent the average of between three and six experiments. N.A., not applicable.
Figure 2. Response of nontransgenic and transgenic T cells to MTV-ORF, LEL, and HYB PRO APCs. (A) T cells were purified from the lymph nodes of C3H/HeN nontransgenic mice and used as responder cells for MLC experiments. Stimulator cells were isolated from the spleens of the age-matched mice of the indicated strains. 5 x 10^6 stimulator cells were used in these experiments. The results shown are representative of 2 of the 12 independent experiments performed. (B) Splenocytes from LEL and MTV-ORF transgenic mice were used as responder cells in MLC assays performed with APCs (5 x 10^6 stimulator cells) isolated from the spleens of LEL, MTV-ORF, HYB PRO transgenic, and C3H/HeN and BALB/c (ALLO response) nontransgenic mice. Shown are the results of one of four experiments.

Table 2. Vβ14+ Primary T Cells Proliferate in Response to HYB PRO Transgenic Splenocytes

| T cells bearing Vβ14 or Vβ6 | CD4/Vβ14 | CD4/Vβ6 |
|-----------------------------|----------|----------|
| Before stimulation          | 7.5      | 9.2      |
| After stimulation           | 35.3     | 0.82     |

* T cells were isolated from the lymph nodes of the nontransgenic C3H/HeN mouse and analyzed by FACS for the percentage of CD4/Vβ14 or CD4/Vβ6 before or after stimulation with the splenocytes isolated from a HYB PRO transgenic mouse. The percentage of CD4+ T cells that are Vβ14+ or Vβ6+ are shown. The data from one of two experiments are shown.

To determine whether the HYB PRO APCs expressed molecules other than the MMTV Sag that could stimulate T cells, lymph node cells from the MTV-ORF and LEL mice were tested for their response to HYB PRO splenocytes (Fig. 2 B). Although the MTV-ORF and LEL T cells responded to allogeneic BALB/c stimulators, they did not respond to the HYB PRO cells. Therefore, even though their spleen cells did not stimulate nontransgenic C3H responder cells, the MTV-ORF mice were functionally tolerant to the MMTV Sag. This agrees with other studies showing a lower threshold for tolerance or deletion than for stimulation (25, 26). These data, taken together with those in Fig. 3, demonstrate that all of the proliferative response of C3H T cells to LEL or HYB PRO APCs was due to Sag recognition.

One possible explanation for the ability of the LEL and HYB PRO splenocytes to present Sag was that the env sequences in these transgenes caused upregulation of sag transcription in these cells relative to the MTV-ORF APCs. To
compare transgene expression among the different strains, a specific probe that distinguished between the 3' end of the HYB PRO, LEL, and MTV-ORF and endogenous MMTV transcripts (Fig. 1) was used for RNAase T1 protection assays. These assays were performed with RNA isolated from the salivary gland of the transgenic strains, since this organ contains very high levels of both endogenous MMTV and MMTV LTR-directed transgene RNA (27). The level of transgene RNA in the HYB PRO transgenic mice was approximately equal to the MTV-ORF transgenic mice (Fig. 4, compare lanes 1 and 2 to lane 3). Similar results were obtained with RNA isolated from spleen and other tissues (data not shown). Moreover, although both strains of LEL transgenic mice deleted their Vβ14+ T cells in vivo and presented the Sag protein in vitro, transgene-specific RNA was not detectable in either strain, indicating that both strains of LEL mice expressed very low levels (Fig. 4, lanes 4 and 5). We have been able to detect expression of the LEL transgene only in the lactating mammary gland of these mice (not shown).

The MTV-ORF mice most likely expressed the highest levels of the sag-specific transcript, since all of the transgene RNA detected in this assay codes for this gene (Fig. 1). In contrast, only one of the three possible viral transcripts produced in the HYB PRO mice encoded the sag gene product (the probe used detected the 3' end of all three viral RNAs: gag/pol, env, and sag) (Fig. 1). Thus, the ability of splenocytes from the MTV-ORF mice to present the C3H Sag was not related to the level of transgene transcription, although we cannot rule out that the level of functional protein expressed is lower in these mice than in the HYB PRO and LEL mice (see below).

These data suggested that there was a contribution to Sag presentation by other genes encoded in the MMTV genome. Since the only difference between the MTV-ORF mice that was shared by the HYB PRO and LEL mice was the presence of the C3H exogenous virus env genes (the gag and pol genes were derived from the Mtv-1 endogenous provirus already found in C3H/HeN mice), our results suggested that the gp52 or gp36 proteins were involved in the processing or presentation of the Sag protein. Because gp52 is the major viral cell surface protein found on infected cells (3), we tested whether it was participating in the Sag stimulation of cognate T cells by adding monospecific anti-C3H exogenous virus-gp52 affinity-purified polyclonal antibody (Fig. 5) to MLC assays in which either LEL or HYB PRO splenocytes were used as APCs. At the highest concentration used, this antibody suppressed by 50–75% the response of nontransgenic responder cells to either LEL or HYB PRO APCs and the level of inhibition was proportional to the amount of antibody present in the assay (Fig. 6, A and B), indicating that gp52 was involved in Sag presentation.

In contrast to the effect seen with the anti-gp52 antibody, neither NGG (Fig. 6 A) nor monospecific antibody directed against the MMTV gp36 Env or p27 virion core proteins (not shown) inhibited the response to LEL or HYB PRO APCs. Moreover, the anti-gp52 antibody did not block the allogeneic response of C3H T cells to BALB/c splenocytes (Fig. 6 B), showing that the inhibition was specific to the Sag response. Preadsorption of the anti-gp52 antibody with nontransgenic C3H/HeN spleen cells did not affect its ability to block Sag recognition by T cells, whereas preincubation with HYB PRO spleen cells completely removed this effect (Fig. 6 C). Thus, the antibody inhibition of the Mls response was specific to recognition of exogenous MMTV gp52 protein and not due to interaction of the antibody with normal T cell or APC cell surface proteins.

There are several possible ways in which the gp52 protein could contribute to the T cell response to the MMTV Sag. Since gp52 is a cell membrane protein, it could interact with the Sag protein and be involved in its transport to the surface. If the gp52 protein were involved in transport, however, it is unlikely that the anti-gp52 polyclonal antiserum would block the T cell response. Alternatively, the Env protein could stabilize cell surface Sag protein expression and decrease its turnover or it could stabilize a Sag/MHC class II complex. We cannot directly address these possibilities.
Figure 6. Inhibition of the Mls response by anti-gp52 antibody. (A) Responder T cells from the lymph nodes of C3H/HeN nontransgenic mice were stimulated with $5 \times 10^6$ splenocytes from LEL no. 1 in the presence of the indicated concentrations of NGG or anti-gp52 antibodies. The results of one of three experiments are shown. (B) T cells isolated from the lymph nodes of C3H/HeN nontransgenic mice were stimulated with $5 \times 10^6$ LEL no. 1, $2 \times 10^6$ HYB PRO, or $10^6$ BALB/c (ALLO) splenocytes in the presence of the indicated concentrations of anti-gp52 antibody or NGG and the percent inhibition was calculated as described in Materials and Methods. The mean and SEM of three experiments for the LEL and BALB/c mice are shown; one experiment for the HYB PRO mouse is shown. (C) T cells from the lymph nodes of C3H/HeN nontransgenic mice were stimulated with $5 \times 10^6$ splenocytes from LEL no. 1 in the presence of the indicated concentrations of anti-gp52 antibody (gp52) or gp52 antibody preabsorbed with nontransgenic (gp52 [C3H/HeN]) or HYB PRO transgenic (gp52 [HYB PRO]) splenocytes. The results of one of two experiments are shown.

since there are no immunological reagents for the C3H exogenous Sag protein available that would allow measurement of cell surface levels, as has been done for endogenous Sags (28–30). However, because of the extremely low levels of Sag-encoding RNA in the LEL mice, whose APCs can induce a T cell response, relative to the MTV-ORF mice, whose APCs cannot, it seems unlikely that there was more Sag protein expressed in the former. All of these models could result in an increased density of the Sag ligand to levels that activate T cells.

Another possibility is that the gp52 protein functions as a costimulatory signal. It is now known that T cell receptor recognition of conventional antigen peptides alone does not provide sufficient stimulus to induce a T cell-mediated immune response and that additional cell–cell interactions between activated T cells and APCs are required (31). Such stimuli are provided by the interaction of APC molecules such as B7 with the CD28 receptor found on the T cell surface (32) and heat-stable antigen and its unknown receptor (33). Costimulation through these molecules leads to enhanced cytokine production by primary T cells and the subsequent proliferation of both T and B cells. Since the gp52 Env protein is found on the membrane of infected cells (4), it is possible that it could provide a costimulatory signal during Sag presentation by interacting with a molecule expressed in the responding primary T cells or hybridomas. If gp52 does participate in the Mls response in this manner, our results indicate that there are different requirements for the presentation of Sag and conventional antigens to T cell hybridomas, since the latter are thought not to require costimulatory signals (34).

In contrast to the results presented here, a number of investigators have shown that the sag gene alone transfected into APCs, such as immortalized B cell lines, is presented to T cell hybridomas expressing suitable receptors (35–37). There are several possible reasons for this discrepancy. First, in the transfection experiments, strong promoter/enhancer regions from either cytomegalovirus or the β-actin gene were used to drive sag expression and the level of antigen presented in transfected cells may be much greater than in primary splenocytes (28). Second, the APCs used in the transfection assays contain several endogenous MMTVs that are expressed and could have functional env genes that complement the transfected sag gene. It is interesting to note, one group has reported complementation of presentation of a transfected sag gene by a coexpressed env gene when the Moloney murine leukemia virus LTR was used to direct expression (36).

These results show that although the sag gene product alone is able to cause the specific deletion of Vβ14+ T cells and the induction of tolerance in vivo, it is not sufficient to induce the Mls response in vitro. Interestingly, although the LEL mice expressed the lowest transgene levels, they had approximately the same level of Vβ14+ T-cell deletion as the HYB PRO and MTV-ORF mice (Table 1). We have previously shown that the level of cognate T cell deletion was...
proportional to the level of sag transcription among different MTV-ORF mice (8). It appears, therefore, that the presence of Env may also result in better Sag presentation and cognate T cell deletion in vivo, as well as in in vitro MLC assays. It has recently been shown that B cells can be infected by MMTV and can transfer virus to T cells (38). The cell surface of such B cells or other APCs must therefore contain the protein products of both the sag and env genes. The use of the Env protein as an additional signal by infected APCs could lead to greater stimulation of responding cells and greater levels of infection. This mechanism of stimulation of T cells by MMTV-infected APCs may have evolved as an efficient mechanism for transferring virus, especially at the early stages of infection when the total number of infected cells is very low.

We thank P. Wright for expert technical assistance, J. P. Dudley for comments on the manuscript, and J. Kappler and P. Marrack for the Kox 15-8.3 cells. We gratefully acknowledge the expertise and equipment provided by the Flow Cytometry Laboratory of the Research Resources Center of the University of Illinois at Chicago.

This work was supported by United States Public Health Service grant CA-45954. T. V. Golovkina was supported by a Cancer Research Institute postdoctoral fellowship and A. Chervonsky was supported by an award from the Johnson and Johnson trustees and United States Public Health Service grant AI-14579. C. A. Janeway, Jr., is an Investigator of the Howard Hughes Medical Institute.

Address correspondence to Susan R. Ross, Department of Biochemistry (m/c 536), University of Illinois, 1819 W. Polk St., Chicago, IL 60612.

Received for publication 8 September 1993 and in revised form 12 October 1993.

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