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Distinct Domains of M-T2, the Myxoma Virus Tumor Necrosis Factor (TNF) Receptor Homolog, Mediate Extracellular TNF Binding and Intracellular Apoptosis Inhibition

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The myxoma virus tumor necrosis factor (TNF) receptor homolog, M-T2, is expressed both as a secreted glycoprotein that inhibits the cytolytic activity of rabbit TNF-α and as an endoglycosidase H-sensitive intracellular species that prevents myxoma virus-infected $CD4^+$ T lymphocytes from undergoing apoptosis. To compare the domains of M-T2 mediating extracellular TNF inhibition and intracellular apoptosis inhibition, recombinant myxoma viruses expressing nested C-terminal truncations of M-T2 protein were constructed. One mutant, ΔL113, containing intact copies of only two cysteine-rich domains, was not secreted and was incapable of binding rabbit TNF-α yet retained full ability to inhibit virus-induced apoptosis of RL-5 cells. Thus, the minimal domain of intracellular M-T2 protein required to inhibit apoptosis is distinct from that required by the extracellular M-T2 for functional TNF-α binding and inhibition. This is the first report of a virus-encoded immunomodular protein with two distinct antiimmune properties.

Poxviruses form a family of large, double-stranded DNA viruses that includes the orthopoxviruses variola virus and vaccinia virus and the leporipoxvirus myxoma virus (17). Poxviruses replicate exclusively within the cytoplasm of infected cells, and the genes essential for virion structure and replication tend to cluster within the central region of the genome, whereas genes encoding virulence factors that are dispensable for growth in tissue culture are often found within the terminal regions (4, 33). Myxoma virus provides a useful model to examine poxvirus pathogenesis in that it causes a systemic immunosuppressive disease, myxomatosis, with a well-defined pathology in the European rabbit, *Oryctolagus cuniculus* (7, 16).

The M-T2 protein of myxoma virus has amino acid sequence homology with the tumor necrosis factor (TNF) receptor superfamily, including the p55 and p75 TNF receptors, Fas, CD40, CD30, and the lymphotoxin β receptor (6, 28, 30). Homology between secreted M-T2 and the membrane-associated TNF receptor family members is limited to the N-terminal ligand binding region and is characterized by four conserved cysteine-rich domains (CRDs) (34). In addition to mediating immune and inflammatory responses, TNF-α is directly cytotoxic to certain virally infected cells (1, 32, 36). Previously, we have shown that M-T2 is an important virulence factor for myxoma virus infection (34) and that secreted M-T2 protein specifically binds and inhibits rabbit TNF-α with an affinity comparable to that of TNF with cellular receptors (24, 25, 34). In addition to inhibiting extracellular rabbit TNF-α, recent data demonstrate that M-T2 expression may be required for productive viral infection of rabbit T lymphocytes (12).

Apoptotic death is an innate protective mechanism of the host against viral infection (5, 22), and many viruses have adapted to the host's apoptotic response by encoding factors which either directly inhibit the apoptotic cascade or interfere with the apoptotic signal that initiates the death pathway within infected cells (27, 35). Myxoma virus replication in lymphoid cells is thought to be the principal mechanism by which the virus is able to disseminate via the lymphoreticular system and establish infections at secondary sites within the host and is an important determinant for the extreme virulence of the virus in infected rabbits (14). Two myxoma virus proteins, M-T2 and M11L, have been shown to be involved in the inhibition of apoptosis in virus-infected RL-5 cells ($CD4^+$ T-cell lymphoma cells), and both are required for viral replication within these cells (12). The M11L gene product has also been shown to be important for viral replication within primary rabbit splenocytes (21). Viruses in which the M11L or *M-T2* gene is inactivated are unable to cause full-blown myxomatosis in infected rabbits (21, 34). In the case of the M-T2-inactivated virus, there was a reduction in the number and severity of lesions at secondary sites (34). These different gene products may thus be important for productive viral replication within and dissemination via subpopulations of infected rabbit lymphoid cells.

Thus, the abilities of M-T2 protein to both inhibit extracellular TNF-α and prevent apoptosis of infected lymphocytes may be important factors in virus pathogenesis. Although M-T2 protein was first characterized as a secreted viroceptor (15, 34), the antiapoptotic activity of M-T2 protein could not be duplicated by addition of exogenous M-T2 protein to T cells infected with M-T2-knockout myxoma virus (12). Here we investigate the expression of M-T2 protein in myxoma virus-infected cells and study its inhibitory properties. We have also tested a series of recombinant myxoma virus constructs that express nested C-terminal truncations of M-T2 for the ability to inhibit apoptosis from an intracellular location in virus-infected lymphocytes and show that the CRD domains of M-T2 required for inhibition of extracellular TNF-α are different from those required for apoptosis inhibition in infected T lymphocytes.

MATERIALS AND METHODS

Viruses and cells. VV-601, a derivative of vaccinia virus (strain WR) with the *Escherichia coli lacZ* gene inserted into the vaccinia virus thymidine kinase locus (13), VV-MT2, constructed to overexpress and secrete the M-T2 protein (24), and VV-T2RaTNF, designed to overexpress and secrete rabbit TNF-α by using the M-T2 signal sequence (25), have been described previously. vMyxlac, a

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derivative of myxoma virus containing the *E. coli lacZ* gene inserted at an innocuous site between the myxoma virus growth factor and M9 genes (21), vMyxt2g, in which both copies of the *M-T2* gene are disrupted by insertion of an *E. coli* guanine phosphoribosyltransferase (*gpt*) cassette (34), and vMyxt2r, in which both copies of the *M-T2* genes in vMyxt2g are reconstituted to wild type (12), are described elsewhere. Viruses were propagated in BGMK cells (from S. Dales) cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco). Rabbit RL-5 cells ($CD4^+$ T-cell lymphoma cells obtained from the NIH AIDS Research and Reference Reagent Program) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum; mouse L929-8 cells (from L. Guilbert) were grown in Iscove's modified Eagle's medium supplemented with 10% fetal bovine serum.

Time course of M-T2 synthesis and secretion from myxoma virus-infected cells. BGMK cells (10^6 cells/well of a six-well dish) or RL-5 cells (2×10^6 cells) were infected with vMyxlac or vMyxt2g at a multiplicity of infection (MOI) of 20. At the times indicated, cells were washed with phosphate-buffered saline (PBS) and incubated in 0.5 ml of serum-free, Met-free, Cys-free DMEM containing 100 μCi of ^{35}S Express protein label ([^{35}S]Met/Cys mixture; Dupont NEN), after which the medium containing secreted, radiolabeled M-T2 was collected, and the cells were washed once in PBS and lysed with Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-Cl [pH 8.0]) containing 0.05% (vol/vol) sodium dodecyl sulfate (SDS). Supernatants and lysates were precleared by incubation with protein A (protein A–Sepharose CL-4B; Pharmacia) beads (30% [vol/vol] in NP-40 lysis buffer, 1 h, 4°C), after which the beads were pelleted by centrifugation ($10{,}000 \times g$, 1 min). To immunoprecipitate M-T2 protein, 1 μl of affinity-purified rabbit anti-M-T2 antiserum was added to either cell supernatants or cell lysates, and the mixture was incubated for 1 h at 4°C, and 30 μl of protein A beads was added. The antibody-antigen reaction mixture was incubated for a further 30 min at 4°C; then the beads were collected by centrifugation at $10{,}000 \times g$ for 1 min and the immune complexes were washed three times with NP-40 lysis buffer, resuspended in 50 μl of Laemmli sample buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were fixed for 1 h in 5% acetic acid–15% methanol, impregnated with En^3Hance (Dupont), dried, and analyzed by fluorography. To immunoprecipitate M-T7, supernatants from infected cells were incubated with 1 μl of affinity-purified rabbit anti-M-T7 antibody (18), and the M-T7 was protein immunoprecipitated as described above.

Pulse-chase analysis of intracellular and secreted M-T2 protein. BGMK cells (10^6) or RL-5 cells (2×10^6) were infected with vMyxlac or vMyxt2g at an MOI of 10. Thirty minutes prior to the labeling time, cells were washed with PBS and incubated in 0.5 ml of serum-free, Met-free, Cys-free DMEM. Cells were then incubated in 0.5 ml of serum-free DMEM containing 100 μCi of ^{35}S Express protein label, after which they were washed with PBS and incubated in DMEM or RPMI 1640 for increasing amounts of time. Supernatants and cell lysates were harvested; M-T2 protein was immunoprecipitated and resolved by SDS-PAGE as described above.

Endoglycosidase H (endo-H) analysis of intracellular and secreted M-T2 protein. Protein A beads containing immunoprecipitated M-T2 protein or M-T2 C-terminal deletions were washed with PBS and divided into two aliquots. Protein was denatured by boiling samples for 10 min in 20 μl of 0.5% SDS–1% β-mercaptoethanol, after which 2 μl of 500 mM sodium citrate (pH 5.5) and, to one of the aliquots, 1,000 U of Endo H_f (New England BioLabs) were added. Samples were incubated for 1 h at 37°C, and reaction products were analyzed by SDS-PAGE followed by fluorography or Western analysis as described above.

Northern blotting of M-T2 mRNA. BGMK cells were infected with vMyxlac or vMyxt2g at an MOI of 20, and RNA was extracted from infected cells at various time points postinfection, using TRIzol reagent (Life Technologies). Early RNA was prepared from infected cells cultured in the presence of arabinosylcytosine (40 μl/ml). Cells were lysed with TRIzol reagent (1.0 ml/10^6 cells), 0.2 ml of chloroform was added, and samples were centrifuged ($12{,}000 \times g$, 15 min, 4°C). The aqueous phase, containing RNA separated from cellular DNA and proteins, was collected, and the RNA was precipitated with 0.5 ml of isopropanol, pelleted by centrifugation ($12{,}000 \times g$, 10 min), and redissolved in 0.5% SDS. RNA samples (3 μg) were denatured by incubation at 55°C for 15 min in morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) containing 50% deionized formamide and 20% formaldehyde, resolved by electrophoresis through a 1% agarose gel containing 1× MOPS buffer, 2% formaldehyde, and 0.5 μg of ethidium bromide per ml, and transferred to nitrocellulose by capillary action. Nitrocellulose membranes were baked in a 80°C oven for 2 h and then prehybridized and hybridized in 50% formamide–0.75 M NaCl–50 mM NaH_2PO_4–5 mM EDTA–2× Denhardt's solution–0.1% SDS. For hybridization, an M-T2 probe was prepared by random priming using a 0.9-kb M-T2 PCR product with [α-^{32}P]dCTP (Dupont NEN) and random primer (Boehringer Mannheim). Blots were washed in 15 mM NaCl–1.5 mM sodium citrate–0.1% SDS at 55°C, and bound probe was visualized by autoradiography.

TNF cytolysis assays. The ability of M-T2 protein to inhibit the cytolysis of L929-8 cells by rabbit TNF-α was determined by incubating actinomycin D-sensitized L929-8 cells with various concentrations of rabbit TNF-α and 2% RPMI 1640 medium containing 500 nM purified M-T2 protein for 18 h at 37°C, and the cell viability was measured by neutral red uptake as previously described (3, 24). L929-8 cells incubated with medium alone were used as a reference for maximum viability, and L929-8 cells incubated in the presence of 100 nM mouse TNF-α were used as a reference for maximum cytolysis. Results are expressed as the percentage viability, the mean of quadruplicate samples calculated by using the formula $[(OD_{experimental} - OD_{maximum\ killing})/(OD_{maximum\ viability} - OD_{maximum\ killing})] \times 100$, where OD is optical density.

Generation of C-terminal M-T2 deletions in recombinant myxoma viruses. All recombinant DNA techniques were carried out according to standard procedures. Myxoma viruses containing C-terminal deletions in each of the two copies of the *M-T2* gene were created by homologous recombination with plasmid DNA containing the desired truncations followed by an in-frame stop codon, the *E. coli gpt* gene to exert dominant selection pressure, and 3′ flanking *M-T2* sequences. The strategy used to generate C-terminal truncations in the *M-T2* open reading frame (ORF) in vaccinia virus vectors is described elsewhere (23). To construct the deletion plasmids, the 218-bp *Sal*I/*Pst*I fragment encoding 3′ and downstream *M-T2* gene sequences was subcloned into the *Sal*I/*Pst*I sites of pUC19, generating plasmid pUC-MT2-3′. The *Hin*dIII site within the multiple cloning region was destroyed by digestion with *Hin*dIII, flush-end repaired with *E. coli* polymerase I Klenow fragment, and blunt-end ligated. A *Hin*dIII site upstream of the *Bam*HI and *M-T2* 3′ sequence was generated by subcloning in complementary oligonucleotides (5′GATCCCCGGGTACCCAAGCTGAGCT and 5′C AGCTTGGGTACCCGGG) into the *Sst*I and *Bam*HI sites. A 940-bp *Sal*I fragment from p7.5 gptA (34) containing an *E. coli gpt* cassette downstream of the p7.5 poxvirus promoter was subcloned into the *Sal*I site, oriented with *gpt* 3′ sequence proximal to the *M-T2* 3′ sequence, generating pUCgpt/MT2-3′. The M-T2 C-terminal deletions followed by an in-frame stop codon were subcloned from plasmids pMTN-ΔL113 to pMTN-ΔD303 (23) as *Hin*dIII/*Bam*HI restriction fragments into the *Hin*dIII/*Bam*HI sites of pUCgpt/MT2-3′, yielding plasmids pUCMT2ΔL113 to pUCMT2ΔD303.

Recombinant myxoma viruses were constructed by using the pUC-based pUCMT2Δ plasmids as vectors by infecting BGMK cells with parental vMyxlac virus and, at 5 h postinfection, transfecting cells with a Lipofectin (GibcoBRL)-DNA (1 μg/1 μg) mixture according to the manufacturer's instructions. After 2 days, cells were harvested and passaged three times with selection medium (25 μg of myxophenolic acid, 20 μg of xanthine, and 15 μg of hypoxanthine per ml), white foci were selected, and individual recombinant foci were plaque purified three times. PCR was used to check for correct insertion of DNA into both copies of the *M-T2* gene, using appropriate primers flanking the gene.

Coimmunoprecipitation of rabbit TNF-α with M-T2 and the C-terminal deletion mutants. To prepare ^{35}S-labeled rabbit TNF-α, BGMK cells (5×10^6) were infected with VV-T2RaTNF at an MOI of 10; 4 h postinfection, cells were washed with PBS and incubated in 2.5 ml of serum-free, Met-free, Cys-free DMEM containing 500 μCi of ^{35}S Express protein label for 18 h, after which the medium was collected and precleared by incubation with protein A beads. The M-T2 leader sequence fused to rabbit TNF-α ligand sequences allowed for efficient secretion of mature TNF-α. BGMK cells (10^6 cells) were infected with vMyxlac, vMyxt2g, or the different viruses expressing the various M-T2 mutant proteins at an MOI of 10; after 24 h, supernatants were harvested and cells lysed with NP-40 lysis buffer. Supernatants from the equivalent of 2×10^5 VV-T2RaTNF-infected cells, containing [^{35}S]Met/Cys-labeled rabbit TNFα, were coincubated for 30 min at 4°C with combined supernatants and lysates from 10^6 infected cells. The different M-T2 truncations together with associated proteins were immunoprecipitated with 1 μl of affinity-purified rabbit anti-T2 antiserum, the immune complexes were washed three times with NP-40 lysis buffer, and the immunoprecipitates were resolved by SDS-PAGE as described above. As a control, rabbit TNF-α was immunoprecipitated from supernatants from [^{35}S]Met/Cys-labeled, VV-T2RaTNF-infected cells with 1 μg of purified goat anti-rabbit TNF-α antibody (Pharmingen) and collected on protein A beads as described above.

Replication of myxoma viruses encoding M-T2 C-terminal deletion mutants. A total of 10^6 BGMK or RL-5 cells were adsorbed for 1 h at 37°C with vMyxlac, vMyxt2g (M-T2 knockout), myxoma virus strain Lausanne (vMyx), vMyxt2r (M-T2 revertant), or the different myxoma viruses expressing M-T2 C-terminal truncations at an MOI of 1 (50% of cells infected). Cells were then washed once with PBS, incubated in medium for 36 h at 37°C, removed with 0.15 M NaCl–0.015 M sodium citrate, pelleted, and resuspended in 200 μl of 10 mM Tris-Cl (pH 8.0)–2 mM $MgCl_2$. Virus was released from cells by three freeze-thaw cycles, and virus titers were determined by plaque assay on BGMK cells following staining of fixed monolayers with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) or 0.1% crystal violet as described previously (21). To determine the effect of rabbit TNF-α on virus growth, RL-5 cells were infected with vMyxlac or vMyxt2g in the presence of 50 nM rabbit TNF-α; at the times indicated, the cells were harvested and virus titers were measured as described above.

Quantitation of apoptotic cells by flow cytometry. RL-5 cells (2×10^6 cells) were mock infected or infected with virus at an MOI of 10 for 10 h, after which cells were harvested for terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end staining (TUNEL reaction). Where indicated, infections were carried out in the presence of 500 nM purified M-T2 protein (25). The proportion of cells showing DNA fragmentation was measured by incorporation of fluorescein isothiocyanate (FITC)-12-dUTP (Boehringer Mannheim) into DNA by using TdT (Boehringer Mannheim) and quantitation by flow cytometry as described previously (26). Briefly, cells were washed in PBS containing 1%

serum, fixed in 2% paraformaldehyde in PBS for 30 min at room temperature, washed once with PBS containing 1% serum, and permeabilized with 0.1% Triton X-100–0.1% sodium citrate on ice for 2 min. The permeabilized cells were washed twice with PBS containing 1% serum and incubated in TdT reaction mixture (6 μM FITC-12-dUTP, 60 μM dATP, 1 mM $CoCl_2$, and 25 U TdT in 30 mM Tris-Cl [pH 7.2]–140 mM sodium cacodylate). Nonspecific uptake of label was measured by reacting the cells in the absence of TdT. Reactions were stopped in 20 mM EDTA, and the cells were subjected to flow cytometric analysis on a FACScan (Becton Dickinson), using a 488-nm argon ion laser to excite fluorescent emissions detected through a 530-nm band pass filter. For each sample 10,000 events were collected.

RESULTS

Kinetics of M-T2 protein synthesis and secretion from myxoma virus-infected cells. In addition to being involved in TNF-α inhibition, M-T2 protein plays an antiapoptotic role in viral infection of certain cells. For example, RL-5 cells ($CD4^+$ T cells) infected with the T2-inactivated virus, vMyxt2g, but not the parental virus expressing full-length M-T2 undergo a rapid apoptotic response that precludes productive virus replication (12). The effect of M-T2 appears to be from an intracellular location because exogenous purified M-T2 protein cannot protect against the apoptosis of RL-5 cells induced by vMyxt2g. To determine when during viral infection M-T2 protein was synthesized and processed, BGMK fibroblasts were infected with myxoma virus (vMyxlac), and at various times postinfection, newly synthesized protein was labeled with [^{35}S]Met/Cys for the 2-h interval immediately prior to harvest. Immunoprecipitations from mock- or vMyxt2g-infected cells labeled for 4 h revealed the absence of background proteins immunoprecipitating with the M-T2 antibody (Fig. 1A, lanes 1 and 2). Newly synthesized M-T2 protein was detected at each 2-h time interval throughout a 24-h round of infection, indicating that *M-T2* mRNA was actively translated throughout the virus infection (Fig. 1A, lanes 2 to 14).

To investigate M-T2 secretion, vMyxlac-infected BGMK cells were cultured continuously in the presence of [^{35}S]Met/Cys label, and supernatants were collected at 2-h intervals. Secreted M-T2 protein was first detected in the supernatants collected between 2 and 4 h postinfection (Fig. 1B, lane 4). In each subsequent 2-h interval, up to 12 h postinfection, labeled M-T2 protein was efficiently secreted from infected cells (lanes 4 to 8). After 12 h of infection, M-T2 protein secretion ceased although it was still being actively synthesized within the cell (compare Fig. 1A and B, lanes 9 to 14).

We tested whether the rate at which synthesized M-T2 protein was secreted varied during infection by pulse-chase analysis. Pulse-labeled M-T2 protein was synthesized within a 30-min pulse beginning 2 h postinfection (Fig. 1C, lane 3) but rapidly disappeared from the cell within chase periods of over 1 h (lanes 6 to 8) and was detected in the supernatant fractions (lanes 9 to 11). By 2 h postlabel, most of the protein had been secreted (Fig. 1C, lane 10), and very little intracellular M-T2 was left (lane 6). In contrast, when cells were pulsed with [^{35}S]Met/Cys only 2 h later, i.e., 4 h postinfection, it took much longer for the M-T2 protein produced to be secreted into the supernatant (Fig. 1D, lanes 3 to 11). For example, after a 2-h chase period, approximately half of the labeled M-T2 had been secreted (Fig. 1D), whereas the same 2-h chase period performed only 2 h earlier resulted in almost quantitative secretion (Fig. 1C, lane 6). Thus, within the first 4 h of infection, the rate at which nascent M-T2 protein was secreted became dramatically less efficient, suggesting that viral infection itself might progressively alter M-T2 trafficking and/or secretion as the infection proceeds.

Some of the intracellular M-T2 glycoprotein pool is not terminally processed. Endo H is used as a diagnostic enzyme for glycoproteins that transit through the Golgi complex because it removes N-linked oligosaccharide chains from the immature glycoproteins that predominate in the endoplasmic reticulum and *cis*-Golgi compartments. As the glycoproteins travel through the normal secretory pathways of the cell, processing by α-mannosidase II in the medial Golgi compartments

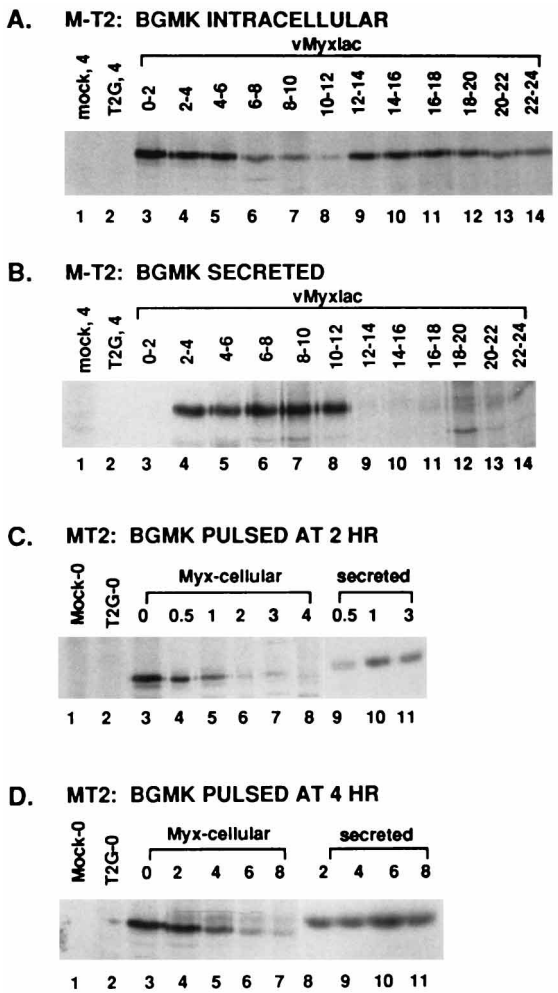

FIG. 1. Kinetics of M-T2 protein synthesis and secretion in infected BGMK cells. (A) Kinetics of intracellular M-T2 synthesis during infection. Shown are immunoprecipitations from mock- or vMyxt2g-infected BGMK cells infected and labeled with [^{35}S]Met/Cys for 4 h (lanes 1 and 2) and from vMyxlac-infected BGMK cells labeled with [^{35}S]Met/Cys 2 h prior to harvest at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h postinfection (lanes 3 to 14). (B) M-T2 protein secretion from infected cells. Shown are immunoprecipitations from supernatants of mock-infected or vMyxt2g-infected BGMK cells infected and labeled with [^{35}S]Met/Cys for 4 h (lanes 1 and 2) or from vMyxlac-infected BGMK cells continuously labeled with [^{35}S]Met/Cys throughout the infection. The medium was then harvested and replaced at 2-h intervals, with harvesting at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h postinfection (lanes 3 to 14). (C) Pulse-chase analysis of M-T2 protein secretion from myxoma virus-infected cells synthesized at 2 h postinfection. Infected BGMK cells were pulsed with [^{35}S]Met/Cys at 2 h postinfection, and M-T2 protein was immunoprecipitated from lysates of mock-, vMyxt2g-, or vMyxlac-infected cells harvested 30 min later (lanes 1 to 3) or chased with cold medium for 30 min and 1, 2, 3, and 4 h (lanes 4 to 8) or from supernatants of vMyxlac-infected cells chased with cold medium for 30 min, 1 h, and 3 h (lanes 9 to 11). (D) Pulse-chase analysis of M-T2 protein secretion from myxoma virus-infected cells synthesized at 4 h postinfection. Infected BGMK cells were pulsed with [^{35}S]Met/Cys at 4 h postinfection, and M-T2 protein was immunoprecipitated from lysates of mock-, vMyxt2g-, or vMyxlac-infected cells harvested 30 min later (lanes 1 to 3) or chased with cold medium for 2, 4, 6, and 8 h (lanes 4 to 7) or from supernatants of vMyxlac-infected cells chased with cold medium for 2, 4, 6, and 8 h (lanes 8 to 11).

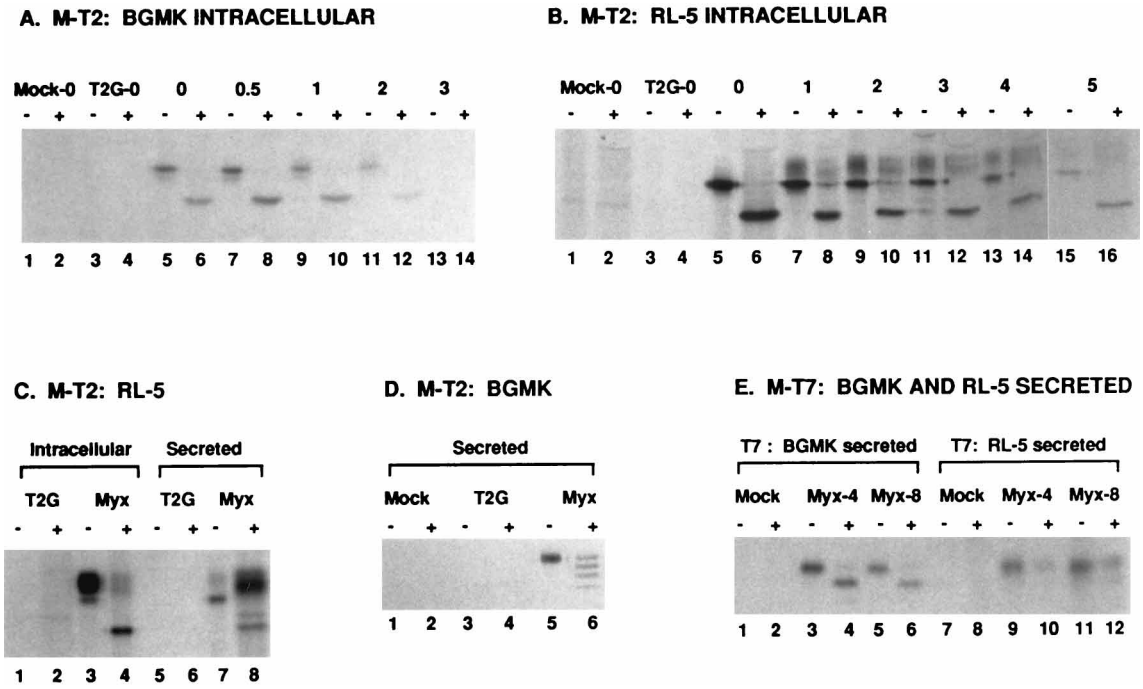

FIG. 2. Endo H sensitivity of intracellular and secreted M-T2 and secreted M-T7 proteins synthesized in infected BGMK and RL-5 cells. (A) Endo H sensitivity of pulse-labeled M-T2 protein synthesized in infected BGMK cells and chased for various amounts of time. At 2 h postinfection, BGMK cells were pulsed with [^{35}S]Met/Cys for 30 min, labeled protein was chased with cold medium for various amounts of time, and M-T2 protein was immunoprecipitated from supernatants and lysates and digested with endo H. Shown are immunoprecipitates from lysates of mock-, vMyxt2g-, or vMyxlac-infected BGMK cells immediately harvested (lanes 1 to 6) or chased with cold medium for 30 min and 1, 2, and 3 h (lanes 7 to 14), undigested (lanes 1, 3, 5, 7, 9, 11, and 13) or digested with endo H (lanes 2, 4, 6, 8, 10, 12, and 14). (B) Endo H sensitivity of pulse-labeled M-T2 protein synthesized in infected RL-5 cells and chased for various amounts of time. At 2 h postinfection, RL-5 cells were pulsed with [^{35}S]Met/Cys for 30 min, labeled protein was chased with cold medium for various amounts of time, and M-T2 protein was then immunoprecipitated from supernatants and lysates and digested with endo H. Shown are immunoprecipitates from lysates of mock-, vMyxt2g-, or vMyxlac-infected RL-5 cells immediately harvested (lanes 1 to 6) or chased with cold medium for 1, 2, 3, 4, and 5 h (lanes 7 to 16), undigested (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or digested with endo H (lanes 2, 4, 6, 8, 10, 12, 14, and 16). (C) Endo H sensitivity of intracellular and secreted M-T2 protein synthesized from myxoma virus-infected RL-5 cells. Infected RL-5 cells were cultured in the presence of [^{35}S]Met/Cys for 16 h, after which the M-T2 protein present in cell lysates and supernatants was immunoprecipitated. Shown are immunoprecipitates from lysates from vMyxt2g-infected (lanes 1 and 2) and vMyxlac-infected (lanes 3 and 4) cells and supernatants from vMyxt2g-infected (lanes 5 and 6) and vMyxlac-infected (lanes 7 and 8) cells, untreated (lanes 1, 3, 5, and 7) and treated (lanes 2, 4, 6, and 8) with endo H. (D) Endo H sensitivity of M-T2 secreted from myxoma virus-infected BGMK cells. Shown are M-T2 immunoprecipitates from supernatants from BGMK cells infected in the presence of [^{35}S]Met/Cys and harvested at 4 h postinfection, mock infected (lanes 1 and 2), vMyxt2g infected (lanes 3, 4), and vMyxlac infected (lanes 5 and 6), untreated (lanes 1, 3, and 5), and treated (lanes 2, 4, and 6) with endo H. (E) Endo H sensitivity of M-T7 produced from infected BGMK and RL-5 cells. BGMK and RL-5 cells, mock or vMyxlac infected, were labeled 4 h prior to harvest, after which secreted M-T7 protein was immunoprecipitated. Shown are M-T7 immunoprecipitates from supernatants from BGMK cells mock (lanes 1 and 2) or vMyxlac (lanes 3 and 4) infected and harvested 4 h postinfection or vMyxlac infected and harvested 8 h postinfection (lanes 5 and 6) from RL-5 cells mock (lanes 7 and 8) or vMyxlac (lanes 9 and 10) infected and harvested 4 h postinfection or vMyxlac infected and harvested 8 h postinfection (lanes 11 and 12), untreated (lanes 1, 3, 5, 7, 9, and 11) and treated (lanes 2, 4, 6, 8, 10, and 12) with endo H.

renders the glycoproteins progressively resistant to cleavage by endo H. Thus, resistance to digestion by endo H is, in general, a marker for glycoproteins that have entered the medial and *trans*-Golgi or later compartments. To study the trafficking of M-T2 protein, the endo H sensitivity of ^{35}S-labeled M-T2 protein, produced in myxoma virus-infected BGMK fibroblasts and RL-5 lymphocytes during a 30-min pulse with [^{35}S]Met/Cys, was examined by immunoprecipitation after various amounts of chase time. These cells were chosen for analysis because BGMK cells are permissive for T2-minus myxoma virus replication, whereas RL-5 cells respond by undergoing apoptosis. In lysates from BGMK cells harvested immediately after the pulse, we observed a 47-kDa endo H-sensitive form of M-T2 protein (Fig. 2A; compare lanes 5 and 6) that is absent as expected from mock- and vMyxt2g-infected cells (lanes 1 to 4). The amount of intracellular labeled M-T2 protein decreased with time of chase: endo H-sensitive intracellular M-T2 protein was observed at 30 min, 1 h, and 2 h postlabel (Fig. 2A, lanes 7 to 12) but had disappeared by the 3-h chase time (lanes 13 and 14). Surprisingly, the M-T2 protein present within the cell could not be chased to an endo H-resistant form, indicating that most of the intracellular M-T2 had not been modified to any appreciable extent by α-mannosidase II present in the medial and *trans*-Golgi compartments.

Figure 2B shows a pulse-chase experiment with myxoma virus-infected CD4$^+$ T lymphocytes (RL-5 cells) pulsed with [^{35}S]Met/Cys at 2 h postinfection. M-T2 protein, migrating at 47 and 57 kDa with and without endo H treatment, respectively, was observed after the 30-min pulse in virus-infected cells but not in mock- or vMyxt2g-infected cells (Fig. 2B, lanes 1 to 6). The intensity of the M-T2 signal within infected cells was gradually reduced over a 5-h chase period (lanes 7 to 16), but intracellular ^{35}S-labeled M-T2 persisted even after a 5-h chase and some of the protein remained endo H sensitive throughout the 5-h chase, indicating that M-T2 was being secreted from RL-5 cells more slowly than BGMK cells. Unlike the results with BGMK cells, however, a higher-molecular-mass diffuse species of M-T2, migrating between 58 and 64 kDa, was observed after a 1-h chase; this species gradually disappeared from the cells and by 5 h was totally absent. This species appeared to be moderately resistant to endo H digestion, as a residual faint diffuse band persisted after endo H

digestion. To investigate this unexpected aspect of M-T2 processing, RL-5 cells were infected in the presence of [^{35}S]Met/Cys for 16 h to uniformly label the cell-associated pool of M-T2 and the accumulated secreted forms. M-T2 protein was then immunoprecipitated from supernatants and cell lysates, digested with endo H, and resolved by SDS-PAGE (Fig. 2C). In cell lysates, harvested 16 h postinfection, two forms of M-T2 protein, a sharp 56-kDa band and a diffuse 59- to 66-kDa band, were immunoprecipitated (lane 3). Upon digestion with endo H, the majority of M-T2 was reduced to a 46-kDa species, with some of the 59- to 66-kDa protein remaining endo H resistant (lane 4). Both species were also observed within supernatants; however, after secretion, the 59- to 66-kDa species of M-T2 remained endo H resistant, whereas the 56-kDa species was endo H sensitive (lanes 7 and 8). Thus, it appears that following infection of RL-5 cells with myxoma virus, two forms of M-T2 protein were secreted. Some of M-T2 secreted was endo H resistant, typical of proteins secreted through the Golgi network, but a proportion of M-T2 remained fully endo H sensitive and was not modified to a mature glycoprotein within the Golgi network.

Figure 2D shows the endo H sensitivity of M-T2 protein secreted from vMyxlac-infected BGMK cells by 4 h postinfection. Upon digestion with endo H, the 56-kDa secreted M-T2 was reduced to bands of 56, 53, 49, and 46 kDa (compare lanes 5 and 6). Since there are four potential N-glycosylation sites within M-T2 protein, this result suggests that the secreted M-T2 was only partially processed to endo H-resistant forms during transit out of the infected fibroblasts. Alternatively, M-T2 protein was processed to a mixture of both hybrid and complex oligosaccharide chains, of which only the complex chains were resistant to endo H (10). It is unlikely that specific N-glycosylation sites within M-T2 were uniquely resistant to α-mannosidase II modification because of protein conformation or interactions with other proteins, since then only one partially endo H resistant species would have been expected.

Because of these surprising results indicating an endo H-sensitive, secreted form of M-T2, we examined another secreted myxoma virus protein, M-T7 (the gamma interferon receptor homolog), to determine if it was similarly endo H sensitive. Figure 2E shows M-T7 protein immunoprecipitated from supernatants of myxoma virus-infected BGMK cells (lanes 3 to 6) or RL-5 cells (lanes 9 to 12) compared to controls from supernatants from mock-infected cells (lanes 1, 2, 7, and 8). The majority of M-T7 protein secreted from infected BGMK cells harvested at 4 and 8 h postinfection was fully endo H sensitive, although some endo H-resistant protein was observed (lanes 4 and 6). In contrast, the majority of M-T7 secreted from RL-5 cells was completely endo H resistant (lanes 10 and 12). Thus, like M-T2, M-T7 was secreted from infected BGMK cells as both endo H-sensitive and endo H-resistant glycoforms, but unlike M-T2, M-T7 was fully processed to endo H-resistant forms in infected RL-5 cells. We conclude that in myxoma virus-infected T lymphocytes, which respond to the absence of M-T2 expression by causing an apoptosis cascade that aborts the virus infection, two distinct glycoforms of M-T2 are secreted and there is an intracellular pool of M-T2 that does not become modified by the standard late Golgi processing pathway. This conclusion suggests the possibility that some intracellular M-T2 protein is diverted into non-Golgi pools during myxoma virus infection of T cells, or else a subset of M-T2 protein is incompletely processed in the late Golgi complex compared to M-T7.

M-T2 gene transcription in myxoma virus-infected cells. Since M-T2 protein was actively synthesized at both early and late times of infection, we investigated M-T2 steady-state mes-

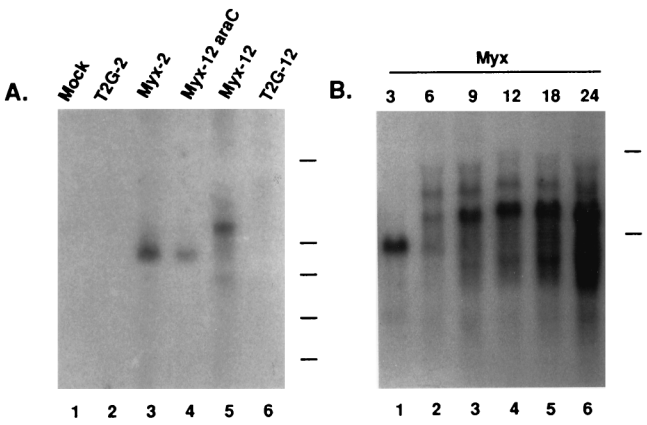

FIG. 3. Northern analysis of M-T2 mRNA in infected cells. (A) BGMK cells were mock, vMyxt2g, or vMyxlac infected in the absence or presence of arabinosylcytosine for various amounts of time, after which RNA was prepared and analyzed by Northern blotting, probing with a 0.9-kb radiolabeled M-T2 probe as outlined in Materials and Methods. RNA prepared from cells mock (lane 1), vMyxt2g (lane 2), and vMyxlac (lane 3) infected for 2 h, vMyxlac infected for 12 h in the presence and absence of arabinosylcytosine (lanes 4 and 5), and vMyxt2g infected for 12 h (lane 6) is shown. Size standards (6.3, 2.4, 1.6, 1.0, and 0.6 kb) are indicated. (B) RNA prepared from vMyxlac-infected BGMK cells harvested at 3, 6, 9, 12, 18, and 24 h postinfection (lanes 1 to 6, respectively) is shown. rRNAs migrating at 6.3 and 2.4 kDa are indicated.

sage levels present at these times by Northern analysis. DNA sequences immediately upstream of the *M-T2* ORF shows all of the important features of an early poxvirus gene, expressed prior to viral DNA replication (34). Moreover, this sequence is very similar to the sequence immediately 5′ of the *T2* ORF in the closely related leporipoxvirus Shope fibroma virus (SFV), *S-T2*, which has been shown to be regulated as an early/late gene (11, 34). However, an unusual array of nine related tandem repeats, 12 to 20 nucleotides long, is present immediately upstream of the *M-T2* ORF, between nucleotides −52 and −182, which is absent in the SFV sequence (34), that could affect expression of M-T2. Figure 3A shows a Northern blot of RNA prepared from myxoma virus-infected BGMK cells and probed with a 0.9-kb PCR product of *M-T2* coding sequence. Lanes 2 and 6 show the absence of any detectable stable message from cells infected with the M-T2-inactivated virus, vMyxt2g. However, a 2.9-kb message was detected by 2 h in myxoma virus-infected cells (lane 3), which was still present at 12 h when the cells were treated with arabinosylcytosine, an inhibitor of DNA synthesis, confirming that *M-T2* was being expressed as an early gene (lane 4). By 12 h postinfection, the early 2.9-kb transcripts could hardly be detected (Fig. 3A, lane 5), even though M-T2 protein was continuously synthesized throughout the infectious cycle (Fig. 1A). Instead, the M-T2 probe hybridized to more heterogeneously sized messages of 4.9, 3.3, and 2.1 kb from vMyxlac-infected cells (Fig. 3A, lane 5) but not transcripts from vMyxt2g-infected cells (lane 6). These multiple bands resembled the run-on transcripts observed with genes transcribed at late times of poxvirus infection when 3′ terminations of viral mRNA become more random in nature (17). In Fig. 3B, showing a Northern blot of RNA prepared from cells infected with myxoma virus and harvested at various times, the 2.9-kb message was the only message observed hybridizing with the M-T2 probe at 3 h postinfection (lane 1); by 6 h, the amount of 2.9-kb message had dropped dramatically, but the 4.9-, 3.3-, and 2.1-kb *M-T2* transcripts instead predominated (lane 2). The origin of these mRNA species will require rigorous analysis of their 5′ ends to

confirm whether they are expressed as late transcripts from the *M-T2* promoter, but the protein labeling experiments (Fig. 2) indicate that *M-T2* translation continues well into late times of infection and so it is presumed that at least some of these late mRNAs do express bona fide M-T2 protein.

Construction of recombinant myxoma viruses expressing C-terminal deletions of M-T2. Recently, we have constructed a series of vaccinia viruses expressing C-terminal truncations of M-T2 protein and demonstrated that only three of the four N-terminal CRDs are required to bind and inhibit rabbit TNF-α (23). To assess the consequences of these mutations in the myxoma virus background, a series of recombinant myxoma viruses, each expressing one of these nested M-T2 truncations instead of wild-type M-T2 in each copy of the viral terminal inverted repeats, was constructed by homologous recombination into the *M-T2* loci of vMyxlac, using *gpt* as a dominant selection marker. The various myxoma viruses used in this study are diagrammed in Fig. 4A. Figure 4B shows full-length M-T2 protein, outlining the fourfold CRD region, including critical cysteine residues responsible for CRD folding, the N-terminal signal cleavage after G16, and four predicted N-glycosylation sites at N66, N181, N205, and N238. The deletion mutant series begins with ΔD303, in which only the C-terminal 24 amino acids have been removed, and end with ΔL113, with only 34% of M-T2 protein sequence present, and are named according to the first amino acid deleted within the truncation. The deletions ΔL113, ΔN169, and ΔE174 are all within the CRDs; ΔL113 contains only the first two intact CRDs, and ΔN169 and ΔE174 contain the first three intact CRDs. All of the other truncations begin after the four N-terminal CRDs. Note that although the vMyxt2g knockout virus could potentially express the N-terminal 45 amino acids of M-T2, no stable mRNA was detected by Northern blotting for this sequence in infected cells (Fig. 3), and no immunoreactive fragments of M-T2 protein could be detected (data not shown).

M-T2 expressed from myxoma virus recombinants requires three CRDs for binding and inhibition of extracellular TNF-α. To determine whether the various myxoma virus-expressed M-T2 truncations are capable of interacting with rabbit TNF-α, the ability of each of the M-T2 truncations as expressed from these recombinant myxoma viruses to coimmunoprecipitate rabbit TNF-α was tested. [^{35}S]Met/Cys-radiolabeled rabbit TNF-α was prepared and mixed with total proteins from cells infected with the myxoma viruses containing the different C-terminal M-T2 deletions and then immunoprecipitated with affinity-purified anti-MT2 antibody. In Fig. 5 (lane 1), 18-kDa rabbit TNF-α prepared from a recombinant vaccinia vector (VV-T2RaTNF) was immunoprecipitated with a polyclonal anti-rabbit TNF antibody and served as a size marker for rabbit TNF-α. Rabbit TNF-α was not immunoprecipitated by the affinity-purified anti-MT2 antibody when it was combined with proteins from vMyxt2g-infected cells (lane 11), but rabbit TNF-α was efficiently coimmunoprecipitated, as expected, with wild-type M-T2 protein from myxoma virus-infected cells (lane 2). The different C-terminal truncations, ΔN169 to ΔD303, although not secreted from myxoma virus-infected cells, nevertheless were all able to coimmunoprecipitate rabbit TNF-α (lanes 4 to 10) in a fashion identical to that for the equivalent C-terminal truncations of M-T2 expressed from vaccinia virus vectors (23), indicating that if any differences in posttranslational processing of M-T2 exist between myxoma virus and vaccinia virus, they would not be significant for TNF-α binding and inhibition. The ΔL113 variant of M-T2, however, was also intracellular but did not coimmunoprecipitate with rabbit TNF-α (lane 3). This ΔL113 truncation begins within the third CRD repeat and includes only eight amino acids of this repeat (Fig. 4B). The inability of ΔL113 to coimmunoprecipitate rabbit TNF-α is completely in agreement with the results from vaccinia virus-expressed truncations and deletions within M-T2 showing that intact copies of the first three CRDs at the N terminus are critical for TNF-α binding and inhibition whereas intact C-terminal sequences are critical for M-T2 secretion (23).

Host cell restriction of the myxoma virus variants bearing C-terminal truncations of M-T2. Previously, it was shown that RL-5 cells readily undergo apoptosis when infected with vMyxt2g, the T2-inactivated virus, but not when infected with vMyxlac, the parental virus for the vMyxt2g construction, or vMyxt2r, a reconstituted M-T2$^+$ control virus (12). Moreover, the replication of vMyxt2g in RL-5 cells was essentially aborted by the apoptotic response, indicating that expression of M-T2 is important for productive viral propagation within RL-5 cells. Therefore, we investigated whether the growth of the various M-T2 truncations within the context of the myxoma virus background was attenuated in RL-5 cells. Figure 6A shows the virus yields from RL-5 cells infected with vMyxlac, vMyxt2g (T2 knockout), vMyx, vMyxt2r (M-T2$^+$ revertant of vMyxt2g), and the different myxoma virus M-T2 C-terminal truncation viruses. Although the yield of vMyx is lower in RL-5 cells than in BGMK cells by an order of magnitude, the growth of vMyxt2g was further inhibited in RL-5 cells compared to the virus yield from vMyxlac, vMyx, and vMyxt2r, indicating that restriction of vMyxt2g growth in RL-5 cells is due to the disruption in M-T2 (12). When the replication of myxoma viruses expressing each of the C-terminal truncations was examined, all replicated within RL-5 cells to an extent comparable to that of vMyxlac and the other M-T2$^+$ control viruses, indicating that each of the M-T2 truncated proteins expressed permitted productive virus replication. Interestingly, the myxoma virus expressing the ΔL113 truncation, which does not coimmunoprecipitate rabbit TNF-α or protect against TNF-α-induced cytolysis, was able to replicate to levels comparable with those of all of the T2-expressing myxoma viruses in RL-5 cells. This finding further supports the notion that the attenuation of the growth of vMyxt2g in RL-5 cells was not due to TNF-α-induced cytolysis of infected cells. Moreover, we used various methods to examine whether RL-5 cells synthesize and secrete TNF-α and found that no cell-associated or secreted TNF-α could be detected by Western analysis or immunoprecipitation with an anti-rabbit TNF-α antibody, and no secreted TNF-α cytolytic activity was detected (data not shown). Furthermore, when RL-5 cells were infected with vMyxt2g or vMyxlac in the presence of 50 nM rabbit TNF-α (Fig. 6A) in vast excess over the amount of M-T2 protein secreted from infected cells (25), no difference was observed between virus yields when rabbit TNF-α was present or absent, indicating that the apoptosis triggered in vMyxt2g-infected RL-5 cells is not mediated by exogenous TNF-α.

The growth of all of these viruses was also examined in permissive BGMK cells, in which the vMyxt2g virus replicates normally compared to vMyx. As expected, virus yields from cells infected with the different truncations were all comparable, within experimental error (Fig. 6B).

Intracellular M-T2 requires only two CRDs for inhibition of apoptosis in infected T lymphocytes. RL-5 cells were infected for 10 h with the various myxoma viruses and examined for the induction of apoptosis. DNA fragmentation within RL-5 cells, a characteristic feature of apoptosis, was measured by TdT incorporation of FITC-12-dUPT into DNA and quantitated by flow cytometric analysis. As previously shown (12), infection of RL-5 cells with vMyxt2g caused a significant population of cells

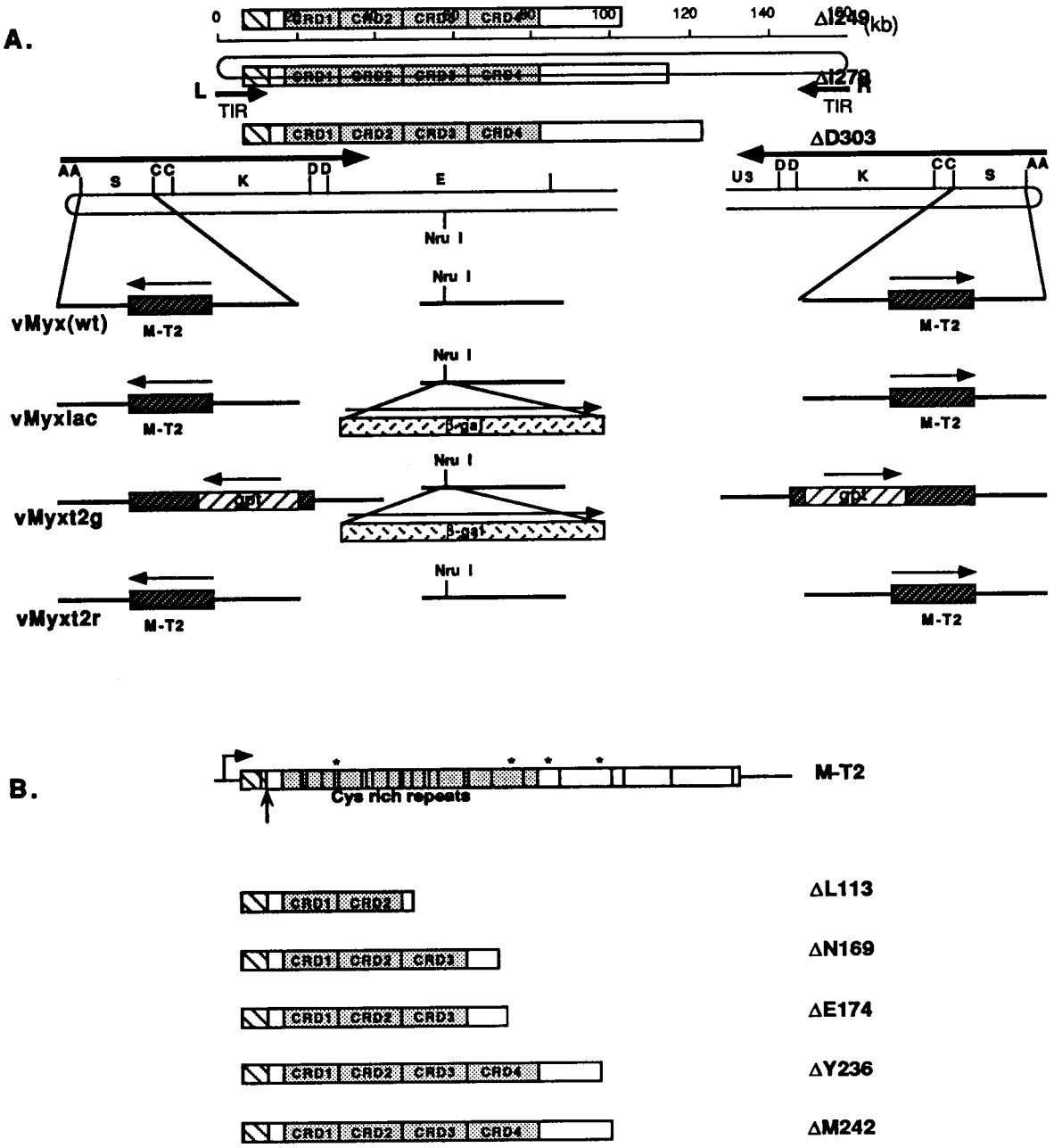

FIG. 4. Structures of the myxoma viruses in which the *M-T2* gene has been inactivated, reconstituted, or truncated. (A) Structure of 160-kDa myxoma virus showing the locations of the *M-T2* genes within each copy of the terminal inverted repeats (TIRs) in wild-type vMyx, vMyx(wt), vMyxlac, and the M-T2 reconstitution virus, vMyxt2r, the insertion of a *gpt* cassette into the *M-T2* gene in vMyxt2g, and the insertion of a β-galactosidase cassette within the *Bam* E fragment in vMyxlac and vMyxt2g. Heavy arrows designate the terminal inverted repeats; light arrows designate ORFs. (B) Structures of the different myxoma virus M-T2 truncations (boxes terminate at the point of each truncation) and full-length M-T2 (lines indicate cysteine residues, shading indicates the four CRDs, asterisks designate N-glycosylation sites, a vertical arrow designates the secretion leader cleavage site, and a bent arrow shows the vaccinia virus synthetic late promoter that controls M-T2 expression).

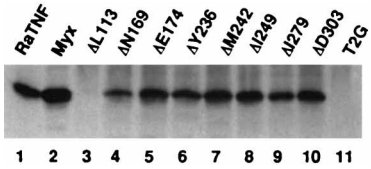

FIG. 5. Coimmunoprecipitation of rabbit TNF-α with full-length M-T2 and the M-T2 truncations expressed from recombinant myxoma viruses. ^{35}S-labeled rabbit TNF-α (RaTNF) present in supernatants from VV-T2RaTNF-infected cells was immunoprecipitated with an anti-rabbit TNF-α antibody (lane 1) or combined with lysates from cells infected with vMyxlac (lane 2), vMyxt2g (lane 11), or myxoma viruses expressing the M-T2 C-terminal truncations ΔL113, ΔN169, ΔE174, ΔY236, ΔM242, ΔI249, ΔI279, and ΔD303 (lanes 3 to 10), immunoprecipitated with an anti-M-T2 antibody as described in Materials and Methods.

to undergo apoptosis (39% [Fig. 7C]), whereas this population was not observed with mock or vMyxlac infection (Fig. 7A and B). When RL-5 cells were infected with vMyxt2g in the presence of 10 nM purified M-T2 protein, a comparable proportion of the cells (37%) were still apoptotic (Fig. 7D), confirming that apoptosis inhibition requires intracellular M-T2 expression (12). As a check to ensure that the exogenous M-T2 protein was still active in this control, the medium containing the purified M-T2 protein was tested before and after culturing with the vMyxt2g-infected RL-5 cells, and the ability of the M-T2 protein to inhibit TNF-α-induced cytolysis of L929-8 cells did not change (Fig. 7M).

RL-5 cells were also infected with the recombinant myxoma viruses expressing the various C-terminal truncations of M-T2. Infection with each of these mutant viruses, including vMyxMT2ΔL113, did not induce any significant apoptosis within RL-5 cells (Fig. 7F to L). This result is in agreement with the previous observation that these viruses were able to replicate within RL-5 cells comparably to vMyxlac (Fig. 6A). Note that because the intact C terminus of M-T2 has been shown to be required for efficient secretion (23), secreted forms of the various deletions were not observed; instead, the M-T2 truncations were present as intracellular, endo H-sensitive proteins, yet apoptosis inhibition was as effective as for the full-length M-T2 protein. Thus, all of these results confirm the contention that M-T2 plays an antiapoptotic role from an intracellular location. Importantly, ΔL113 does not even contain a functional TNF-α-binding domain, yet intracellular expression of this mutant protein fully protects virus-infected RL-5 cells from undergoing apoptosis. We conclude that the protein domain that governs the anti-TNF-α properties of secreted M-T2 is distinct from the minimal domain necessary for the antiapoptotic effects of the intracellular form of the same protein.

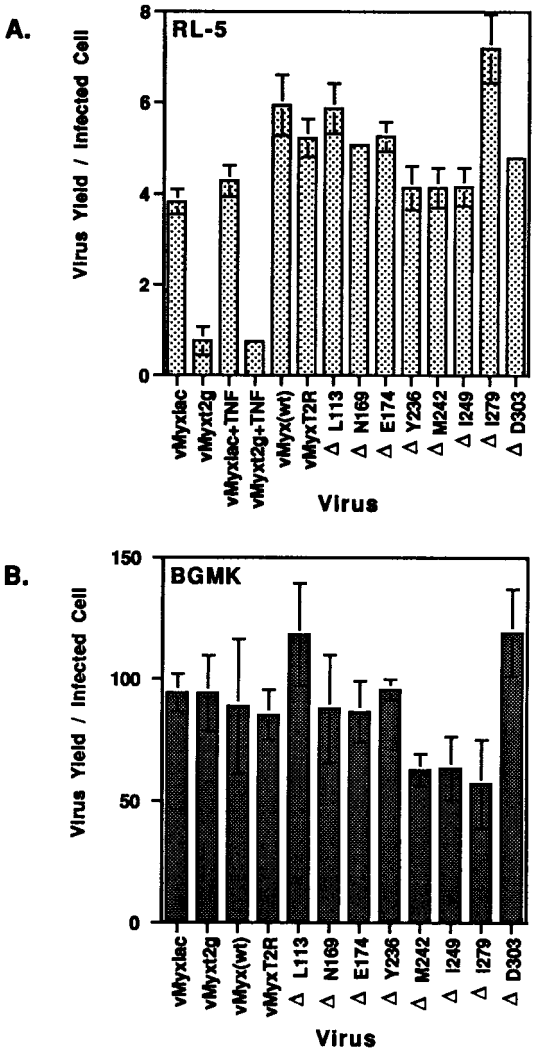

FIG. 6. Growth of the different myxoma constructs expressing full-length M-T2 and M-T2 truncations in RL-5 and BGMK cells. RL-5 (A) and BGMK (B) cells were infected at an MOI of 1; after 36 h, cells were harvested and virus titers were determined by plaque assay as outlined in Materials and Methods. Data are expressed as the virus yield (output PFU/infected cell); each sample was determined in triplicate. vMyxlac- and vMyxt2g-infected RL-5 cells were also continuously cultured in the presence of 50 nM rabbit TNF-α.

DISCUSSION

According to the original viroceptor model of M-T2 action for myxoma virus virulence, the M-T2 protein that is secreted from infected cells binds and sequesters the TNF produced and secreted locally by immune cells in response to viral infection (29, 34). By thus preventing the ligand triggering of TNF receptors, M-T2 protein would inhibit both direct TNF cytolysis of infected cells and other secondary immune responses dependent on TNF. In support of this model, we have shown that secreted M-T2 protein binds rabbit TNF-α in the picomolar range with an affinity comparable to that of TNF-α with cellular receptors and inhibits the cytolytic effect of rabbit TNF-α at molar ratios similar to those observed with soluble versions of the two major TNF receptors (24, 25). Thus, it was unexpected when the ability of M-T2 to inhibit the apoptosis of myxoma virus-infected RL-5 cells could not be mimicked by the addition of exogenous, purified M-T2 to vMyxt2g-infected RL-5 cells at concentrations in vast excess over that required to inhibit the cytolytic activity of rabbit TNF-α (12), suggesting instead that intracellular M-T2 protein was mediating the antiapoptotic effect.

M-T2 expression and secretion were analyzed in BGMK fibroblasts and RL-5 lymphocytes. BGMK cells are permissive for growth of vMyxt2g, but RL-5 cells are nonpermissive due to the supervening apoptotic response to the virus infection. Intracellular forms of M-T2 were readily detected at all times during infection of both BGMK and RL-5 cells, indicating that M-T2 protein was continuously expressed during virus infection and thus would be at least available for binding to intracellular target protein(s) of the host apoptosis pathways. Although M-T2 protein was continuously being synthesized throughout virus infection, the rate of M-T2 secretion was

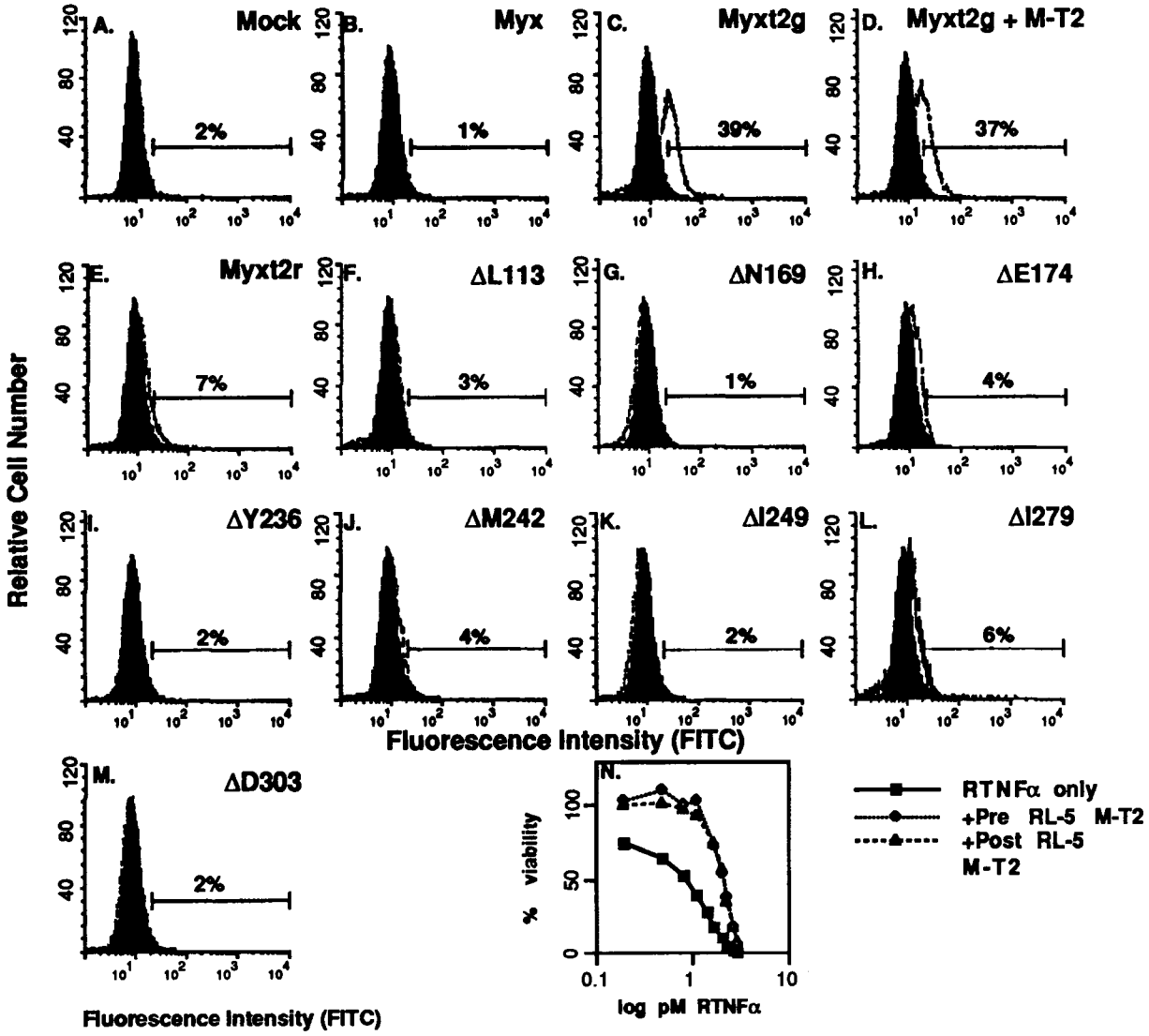

FIG. 7. Quantitation of apoptosis within infected RL-5 cells by FITC-12-dUPT incorporation and flow cytometric analysis. RL-5 cells were infected with virus for 10 h, after which fragmented DNA in apoptotic cells was labelled with FITC-12-dUPT and the amount of fluorescence was quantitated by flow cytometric analysis as described in Materials and Methods. Shown are relative cell numbers versus fluorescence intensities of mock-infected (A) and vMyxlac-infected (B) cells and overlays of vMyxlac-infected (black) and vMyxt2g-infected (C) and vMyxt2g-infected (D) cells infected in the presence of 500 nM M-T2 protein expressed by viruses as indicated in panels E to M. (N) Incubation of M-T2 with the vMyxt2g-infected RL-5 cells used for panels A to M did not affect its ability to inhibit rabbit TNF-α (RTNFα). The percent viability of actinomycin D-sensitized L929-8 cells with increasing TNF-α concentration is shown for TNF alone (squares) or in the presence of 5% RPMI 1640 medium containing 500 nM M-T2 protein before (circles) or after (triangles) culturing of vMyxt2g-infected RL-5 cells as described in Materials and Methods.

significantly reduced as the infection proceeded. Initially, M-T2 protein was rapidly processed and secreted, providing an extracellular source of M-T2 to inhibit local TNF present in the extracellular milieu of infected cells. By 4 h postinfection, however, the rate of M-T2 secretion had dropped dramatically, and at later times, nascent M-T2 protein was fully retained within myxoma virus-infected cells. This decrease could be due to the viral infection altering the host cell secretory machinery, or it could be due to specific retention of M-T2 protein by interaction with host targets within infected cells.

In infected BGMK cell lysates, all of the M-T2 protein observed in the steady-state pools was sensitive to endo H. Although M-T2 protein was secreted from these cells, intracellular M-T2 protein could not be chased to endo H-resistant or partially resistant species, indicating that for M-T2 protein, processing to endo H-resistant forms was relatively inefficient in virus-infected cells. In fact, M-T2 protein secreted from myxoma virus-infected BGMK cells was still mostly sensitive to digestion with endo H, and the cleaved species resolved into at least four distinct species, indicating that a mixture of both complex and hybrid or high-mannose oligosaccharide units was attached at multiple N-glycosylation sites. The N-oligosaccharide chains present on secreted M-T2 protein had been only partially processed to complex oligosaccharide units, suggesting that M-T2 may undergo alternative trafficking pathways in T lymphocytes compared to other viral glycoproteins (e.g., M-T7).

In contrast, both endo H-sensitive and -resistant forms of intracellular M-T2 protein were observed in cell lysates prepared from myxoma virus-infected RL-5 lymphocytes. All of the endo H-resistant M-T2 present within RL-5 cells could be chased to the culture supernatant, whereas only some of the

endo H-sensitive form was secreted during the chase times, indicating it was either retained within the cell or secreted at a much lower rate. In light of the fact that M-T2 protein has an antiapoptotic effect specifically in RL-5 cells, it is possible that the endo H-sensitive form is retained within the cell through interaction with another protein in the apoptotic cascade that results in the diversion of M-T2 away from the secretory pathway. These two forms of M-T2 protein were also observed to be secreted from RL-5 cells. The majority of M-T2 protein in the secreted fraction was resistant to endo H, indicating that its oligosaccharide chains had been processed to mature, fully complex forms. A minor 56-kDa species was also secreted; this species, upon endo H digestion, was reduced to a 46-kDa band, indicating that none of its N-linked oligosaccharide units had been processed to complex sugar chains. This finding suggests that M-T2 from RL-5 cells was processed via two different mechanisms: the majority of M-T2 glycoprotein secreted was processed to complex oligosaccharide units within the Golgi network, whereas a subpopulation of M-T2 did not encounter α-mannosidase II within the Golgi network, presumably because M-T2 protein was complexed with another factor which either prevented oligosaccharide processing by the α-mannosidase II or diverted M-T2 into a distinct cellular compartment.

Significant differences were observed between BGMK and RL-5 cells with respect to both the rates of M-T2 secretion and oligosaccharide processing. Completely endo H resistant forms of M-T2 protein were observed in both cell lysates and supernatants from infected RL-5 cells but not BGMK cells, indicating that there are important differences in oligosaccharide processing between the two cell lines. Variation in sugar processing itself is not unexpected between cells, and modulation of the glycosylation pathway is one mechanism to introduce posttranslational variability and modify protein activities (9, 19, 20). These differences in oligosaccharide processing were not restricted to M-T2 protein. M-T7, the myxoma virus gamma interferon receptor homolog, was also secreted from BGMK cells as an endo H-sensitive species, whereas from RL-5 cells, the N-linked oligosaccharides on M-T7 were fully processed to endo H-resistant complex chains before secretion. A close examination of glycosylation patterns might provide useful information on the intracellular trafficking of M-T2 protein within the cell. In RL-5 cells, the endo H-resistant and -sensitive forms of M-T2 protein may have different functions in preventing infected cells from triggering the apoptotic response.

At the level of gene expression, we confirm here that *M-T2* mRNA was detected at both early and late times of infection and is stable in the presence of arabinosylcytosine, a DNA synthesis inhibitor. The corresponding *S-T2* gene from the related SFV is also expressed at both early and late times of infection, as determined by S1 analysis of transcripts (11). The indication that *M-T2* transcripts are present at both early and late times reaffirms the fact that nascent M-T2 protein is continuously translated within the infected cell.

Although the M-T2 protein N-terminal domain exhibits homology with the CRDs of the TNF receptor superfamily, the C-terminal 132 amino acids are not similar to any known cellular sequence or unrelated viral protein. Each of the M-T2 C-terminal truncations, however, up to and including ΔL113, which includes only the two N-terminal CRDs, was able to fully protect myxoma virus-infected RL-5 cells from undergoing apoptosis, indicating the C terminus of M-T2 is not involved in the antiapoptotic response. The ΔL113 variant of M-T2, which contains only the first two intact CRDs plus eight amino acids of the third CRD, was fully able to prevent apoptosis in RL-5 cells and thus permit productive viral replication of myxoma virus, despite its inability to bind or inhibit TNF-α. Furthermore, all of the various M-T2 C-terminal truncations from ΔL113 to ΔD303 were retained as cell-associated proteins (23), reinforcing the observation that it is the intracellular form of M-T2 protein which inhibits virus-induced apoptosis whereas the secreted form is responsible for extracellular TNF-α ligand inhibition.

Although it remains possible that M-T2 inhibits a downstream effector of an apoptotic signaling cascade, it seems more likely that M-T2, with its homology to the N-terminal domains of the TNF receptor family members, inhibits apoptotic signaling that emanates from a member of the TNF receptor superfamily. However, all of the available evidence indicates that TNF-α ligand itself does not appear to mediate apoptosis within infected RL-5 cells. Since M-T2 protein binds and effectively inhibits rabbit TNF-α (24, 25), we rigorously investigated TNF-α production and sensitivity in RL-5 cells. Rabbit TNF-α did not affect the growth of RL-5 cells in culture, did not affect the replication of myxoma virus within infected RL-5 cells, and could not be detected secreted from infected or uninfected RL-5 cells. Moreover, TNF-α could not be detected within RL-5 cell lysates by Western analysis or by immunoprecipitation (data not shown).

The most plausible rationale for our results is that M-T2 protein heterodimerizes with a member of the TNF receptor superfamily in a dominant-negative fashion to inhibit receptor homodimerization. Since M-T2 protein lacks the appropriate intracellular signaling domains, the receptor–M-T2 complex would be unable to trigger an apoptotic signal through the cytoplasmic death domain. C-terminal truncations of the p55 TNF receptor which act in such a dominant negative fashion to suppress signaling have been described (2, 31). Moreover, a number of dominant interfering mutations within Fas which impair Fas-mediated apoptosis have been found in individuals with autoimmune lymphoproliferative syndrome (8). Under this model of M-T2 action, the ΔL113 truncation would have retained the ability to recognize and form inhibitory heterodimers with a TNF receptor family member despite its inability to bind or inhibit the TNF-α ligand itself. Distinguishing between the hypothetical mechanisms of how intracellular M-T2 prevents the apoptotic response in infected lymphocytes will require cellular localization studies of M-T2 protein and identification of the cellular target protein(s) that associate with it.

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