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Dominant-Negative Mutants of Human MxA Protein: Domains in the Carboxy-Terminal Moiety Are Important for Oligomerization and Antiviral Activity

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Human MxA protein is an interferon-induced 76-kDa GTPase that exhibits antiviral activity against several RNA viruses. Wild-type MxA accumulates in the cytoplasm of cells. TMxA, a modified form of wild-type MxA carrying a foreign nuclear localization signal, accumulates in the cell nucleus. Here we show that MxA protein is translocated into the nucleus together with TMxA when both proteins are expressed simultaneously in the same cell, demonstrating that MxA molecules form tight complexes in living cells. To define domains important for MxA-MxA interaction and antiviral function in vivo, we expressed mutant forms of MxA together with wild-type MxA or TMxA in appropriate cells and analyzed subcellular localization and interfering effects. An MxA deletion mutant, MxA(359–572), formed heterooligomers with TMxA and was translocated to the nucleus, indicating that the region between amino acid positions 359 and 572 contains an interaction domain which is critical for oligomerization of MxA proteins. Mutant T103A with threonine at position 103 replaced by alanine had lost both GTPase and antiviral activities. T103A exhibited a dominant-interfering effect on the antiviral activity of wild-type MxA rendering MxA-expressing cells susceptible to infection with influenza A virus, Thogoto virus, and vesicular stomatitis virus. To determine which sequences are critical for the dominant-negative effect of T103A, we expressed truncated forms of T103A together with wild-type protein. A C-terminal deletion mutant lacking the last 90 amino acids had lost interfering capacity, indicating that an intact C terminus was required. Surprisingly, a truncated version of MxA representing only the C-terminal half of the molecule exerted also a dominant-negative effect on wild-type function, demonstrating that sequences in the C-terminal moiety of MxA are necessary and sufficient for interference. However, all MxA mutants formed hetero-oligomers with TMxA and were translocated to the nucleus, indicating that physical interaction alone is not sufficient for disturbing wild-type function. We propose that dominant-negative mutants directly influence wild-type activity within hetero-oligomers or else compete with wild-type MxA for a cellular or viral target.

The human MxA protein is an interferon-induced 76-kDa GTPase that inhibits the multiplication of several RNA viruses. It blocks replication of orthomyxoviruses (7, 29, 30), rhabdoviruses (31), paramyxoviruses (36, 37), and bunyaviruses (6, 19a). The mechanisms by which MxA is able to block such a diverse group of viruses have not been determined. Furthermore, effector domains as well as viral target structures remain to be identified. Exciting clues come from the observation that recombinant MxA protein produced in *Escherichia coli* inhibits vesicular stomatitis virus (VSV) and influenza A virus RNA synthesis in vitro, suggesting that components of the viral polymerase complex are affected (40, 21).

MxA is a member of the superfamily of large GTP-binding proteins which, as a distinctive feature, exhibit intrinsic GTPase activity (39, 44). Other family members are the dynamins (3, 27, 47), which are involved in endocytosis, two yeast proteins, VPS1 and MGM1, and the plant protein phragmoplastin (10). VPS1 is involved in exocytotic protein transport (35). MGM1 is a protein necessary for the maintenance of the mitochondrial genome (18). The sequence similarities between these proteins are extensive in the amino (N)-terminal moiety containing the conserved GTP-binding domain. However, they do not extend to the carboxy (C)-terminal portions which are thought to comprise specific effector domains (49). A single amino acid substitution near the C terminus was shown to change the antiviral properties of MxA, indicating that this region indeed determines effector specificity (51). Mutations in the GTP-binding element of MxA invariably cause a loss of antiviral activity, suggesting that GTP binding and/or hydrolysis is critical for function (32). Unexpectedly, the C terminus is also required for GTPase activity (39). Schwemmle et al. (39) proposed that the C terminus folds back and interacts with the GTP-binding element to build the enzymatically active center of the protein. Natural and recombinant MxA proteins are known to form high-molecular-weight oligomers (24, 34), but the significance of this process for antiviral function is not yet clear.

Previous attempts to define Mx domains important for antiviral function by mutational analyses of human or mouse Mx proteins have been hampered by the fact that most mutations resulted in a complete loss of antiviral activity (8, 22, 32, 46, 51). Therefore, we chose an alternative approach to identify functional domains in human MxA protein. We cotransfected virus-susceptible cells with wild-type and mutant MxA cDNAs and looked for interfering effects. Here, we describe two mutant MxA proteins that destroy the antiviral function of the wild-type protein. A C-terminal deletion in one of these mutants abolished its interfering effect, suggesting that the C terminus is important for inhibiting wild-type activity. Interestingly, all mutants formed hetero-oligomers with the wild-type protein. Furthermore, we show that a region of 213 amino

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acids is responsible for the intermolecular interaction observed. We propose that the dominant-negative mutants directly influence wild-type activity within these hetero-oligomers or else compete with wild-type proteins for a cellular or viral target.

MATERIALS AND METHODS

Cells. Permanently transfected Swiss mouse 3T3 cells constitutively expressing wild-type human MxA protein (clone 4.5.15) and control 3T3-SVneo cells expressing only the neomycin resistance gene were the same as described previously (31). Cells were grown in Dulbecco's modified minimal essential medium containing 10% fetal calf serum and 0.5 mg of G418 (GIBCO) per ml.

Viruses. Stocks of Thogoto virus (THOV) strain SiAr126 (2), a mammalian cell-adapted variant of influenza A/FPV/Dobson/34 (H7N7) virus, called FPV-B (15), and VSV serotype Indiana (31) were prepared as described previously (11). They contained 8.3×10^7, 3.9×10^8, and 2×10^8 PFU per ml, respectively, as titrated in Vero cells.

Construction of mutant MxA cDNAs and expression vectors. To mark MxA with a tag peptide of eight amino acids (FLAG peptide [12]), we used a MxA cDNA in plasmid pSP64-MxA(*Cla*I), which has a *Cla*I restriction site in place of the original ATG initiation codon (51). The FLAG sense oligonucleotide (5′ CG GTA CCG AAG ATG GAC TAC AAG GAC GAC GAT GA 3′) flanked by *Cla*I adaptors and the complementary antisense oligonucleotide were hybridized, phosphorylated, and inserted into the *Cla*I restriction site of pSP64-MxA(*Cla*I). The resulting construct, pSP64-FLAG-MxA, carried the FLAG sequence as a 5′-terminal extension of the MxA open reading frame.

To create the GTPase-deficient mutant T103A, the codon ACC encoding Thr at position 103 of MxA was converted to the Ala codon GCC by using oligonucleotide 5′ CAG CAC CAG CGG GCA TCT GGC CAC GAT CCC GCT GCC 3′ and a PCR-based site-directed mutagenesis technique, resulting in pSP64-T103A (16, 32). Plasmid pSP64-T103A was then digested with *Ppu*MI and *Nco*I. The resulting fragment, which contained the mutation, was used to replace the corresponding wild-type fragment of pSP64-FLAG-MxA. To generate T103AΔC, a 270-bp fragment located immediately at the 3′ end of T103A was removed. The FLAG-T103A cDNA was digested with *Nco*I, blunted with S1 nuclease, and religated. The different MxA inserts were released by the appropriate restriction enzymes, blunted, and ligated in correct orientation into the unique *Eco*RV site of the eukaryotic expression vector pCL642 (9), yielding plasmids pHMG-FLAG-MxA, pHMG-FLAG-T103A, and pHMG-FLAG-T103AΔC.

MxAΔN was generated by PCR. Plasmid pSP73-MxA, which includes the entire MxA open reading frame, was used as a template. Primer 1 (5′ GAT ATC ATG GAC TAC AAG GAC GAC GAT GAC AAG TAT GGT GTC GAC ATA CC 3′) corresponded to positions 1285 to 1304 of the MxA sequence and contained the FLAG sequence (underlined sequence); primer 2 (5′ GA TAT CAC AGA GTG TGG TTA ACC GGG GAA 3′) was reverse complementary to positions 2188 to 2216. The resulting PCR product contained an *Eco*RV site at both ends and could be directly ligated into the unique *Eco*RV site of pCL642, yielding plasmid pHMG-FLAG-MxAΔN.

MxA(359–572) was also generated by PCR using plasmid pSP73-MxA as a template. Primer 1 (5′ C GGG ATA TCG AAG ATG GAC TAC AAG GAC GAC GAT GAC AAG TAT GGT GTC GAC ATA CC 3′) corresponded to positions 1285 to 1304 of the MxA sequence and contained the FLAG sequence (underlined sequence); primer 2 (5′ CGG GGT ACC CCG TTA CTC CTC CAT GGA AGA GTC 3′) was reverse complementary to positions 1921 to 1938. The resulting PCR product contained an *Eco*RV site at the 5′ end as well as a *Kpn*I site at the 3′ end and could be directly ligated into the *Eco*RV/*Kpn*I-digested vector pCL642, yielding plasmid pHMG-FLAG-MxA(359–572).

All mutations were confirmed by sequencing.

Transfections and establishment of cell lines. For transient transfections, MxA-expressing cells and appropriate control cells were transfected with the FLAG-tagged mutant MxA-cDNA constructs by the calcium phosphate coprecipitation method as previously described (43).

To establish stable cell lines coexpressing wild-type and mutant MxA proteins (3T3-MxA/T103A), the same cells were cotransfected with the desired mutant expression vector and a plasmid for hygromycin resistance (pX343 [25]). Transfected cells were selected in medium containing 10% fetal calf serum, 0.5 mg of G418 per ml, and 150 μg of hygromycin per ml. Resulting clones were screened by indirect immunofluorescence for expression of FLAG-tagged mutant MxA proteins, and positive clones were subjected to further rounds of subcloning in order to select cell lines with uniform expression levels.

Immunofluorescence analysis. Cells were prepared and stained for MxA proteins and viral antigens by indirect immunofluorescence essentially as described previously (5). FLAG-tagged MxA proteins were labeled with a monoclonal mouse antibody anti-FLAG M2 (Integra Biosciences, Fernwald, Germany), which recognizes the first four amino acids of the FLAG sequence. To visualize the nonflagged MxA proteins, monoclonal mouse antibody 2C12 (41) or a polyclonal rabbit antiserum against *E. coli*-expressed histidine-tagged MxA protein was used. To label THOV antigens, hyperimmunized guinea pig antisera kindly provided by P. A. Nuttall, NERC Institute of Virology and Environmental Microbiology, Oxford, England (19), were used. The cells were then stained with dichlorotriazyl amino fluorescein- and/or tetramethylrhodamine isothiocyanate-conjugated goat antibodies which recognize mouse, rabbit, or guinea pig antibodies (Dianova, Hamburg, Germany) and observed with a Reichert-Jung Polyvar or a Leica TCS^{4D} confocal laser scanning microscope equipped for epifluorescence.

Western blot analysis. Total cell extracts were prepared by lysing cells in sample buffer (20). Protein (10 μg/lane) was loaded onto sodium dodecyl sulfate–10% polyacrylamide gels, and Western blot analysis was performed as previously described (6). MxA proteins were detected by using the same primary antibodies as described for immunofluorescence and an alkaline phosphatase-conjugated secondary antibody.

RNA extraction and Northern blot analysis. Detection of viral RNA by Northern blot analysis was performed as described previously (1). The cultures were infected with 5 PFU of THOV per cell and harvested 16 h later. Total RNA was isolated by using RNeasy columns (Qiagen, Hilden, Germany). RNA (20 μg per lane) was subjected to electrophoresis through 1.2% agarose gels containing 3.7% formaldehyde, blotted to Nytran membranes, and hybridized to a radiolabeled probe. The nick-translated probe was derived from the 1.4-kb THOV *NP* gene (50).

Virus plaque assay. Virus plaque assays were performed as described previously (7). Briefly, cell monolayers were infected with 50 to 100 PFU of either THOV (strain SiAr126), influenza A virus (strain FPV-B), or VSV (serotype Indiana). The viruses were allowed to form plaques for 96 h (THOV), 48 h (influenza A virus), and 24 h (VSV) under soft agar.

GTPase assay. Histidine-tagged MxA proteins containing six additional histidine residues at the N terminus were purified from *E. coli* by Ni-agarose affinity chromatography followed by Mono Q ion-exchange chromatography as described previously (40). Purified histidine-tagged MxA (0.04 μg/μl) was incubated at 37°C in a mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10% glycerol, 100 μM GTP, 13 nM [α-^{32}P]GTP, and 200 nM adenosine-5′[β-γ-imido]triphosphate. The reactions were stopped 30 min later with 0.5% sodium dodecyl sulfate and 2 mM EDTA. Samples were spotted onto polyethyleneimine-cellulose thin-layer chromatography plates (Merck, Darmstadt, Germany) and were resolved by running the plates in a solution containing 1 M LiCl and 1 M acetic acid. The dried plates were exposed to X-ray films.

RESULTS

Mutant forms of MxA protein lacking antiviral activity. Three different mutations were produced to analyze the role of distinct MxA domains for antiviral activity (Fig. 1A). First, the threonine at position 103 between the first and second GTP-binding consensus motif was replaced by alanine, generating the mutant protein T103A. When expressed in E. coli and purified to homogeneity, T103A had no GTPase activity (Fig. 1B). Further biochemical characterization revealed that T103A lacked GTP-binding capacity (33). Second, by using a suitable *Nco*I restriction site, a C-terminal deletion mutant of T103A devoid of the last 90 amino acids was produced. This truncated protein will be referred to as T103AΔC. Finally, an N-terminal deletion mutant, MxAΔN, lacking amino acids 1 to 359, was generated, allowing us to study the properties of the C-terminal half of MxA. Mutant or wild-type proteins were N-terminally tagged with the FLAG peptide (12) to facilitate detection by a specific monoclonal antibody. Mouse 3T3 cells were chosen for transfection because they do not express endogenous Mx proteins and have previously been shown to be excellent host cells for monitoring the antiviral activities of human MxA protein (31, 51).

In a first experiment, the FLAG-tagged wild-type and mutant MxA proteins were transiently expressed in mouse 3T3 cells, and their individual anti-THOV activities were determined by double-immunofluorescence analysis. Expression of transfected proteins was assessed with an anti-FLAG antibody, and accumulation of viral antigens was visualized with a THOV-specific antiserum. Figure 2 shows that all mutant proteins were detected in the cytoplasm, as was wild-type MxA. The C-terminal deletion mutant T103AΔC showed a subcellular distribution similar to the characteristic diffuse granular staining pattern of the wild-type protein. In contrast, the full-length T103A mutant and the C-terminal half of MxA formed larger aggregates (Fig. 3 and 4). As expected (7), no THOV antigens accumulated in infected cells expressing FLAG-

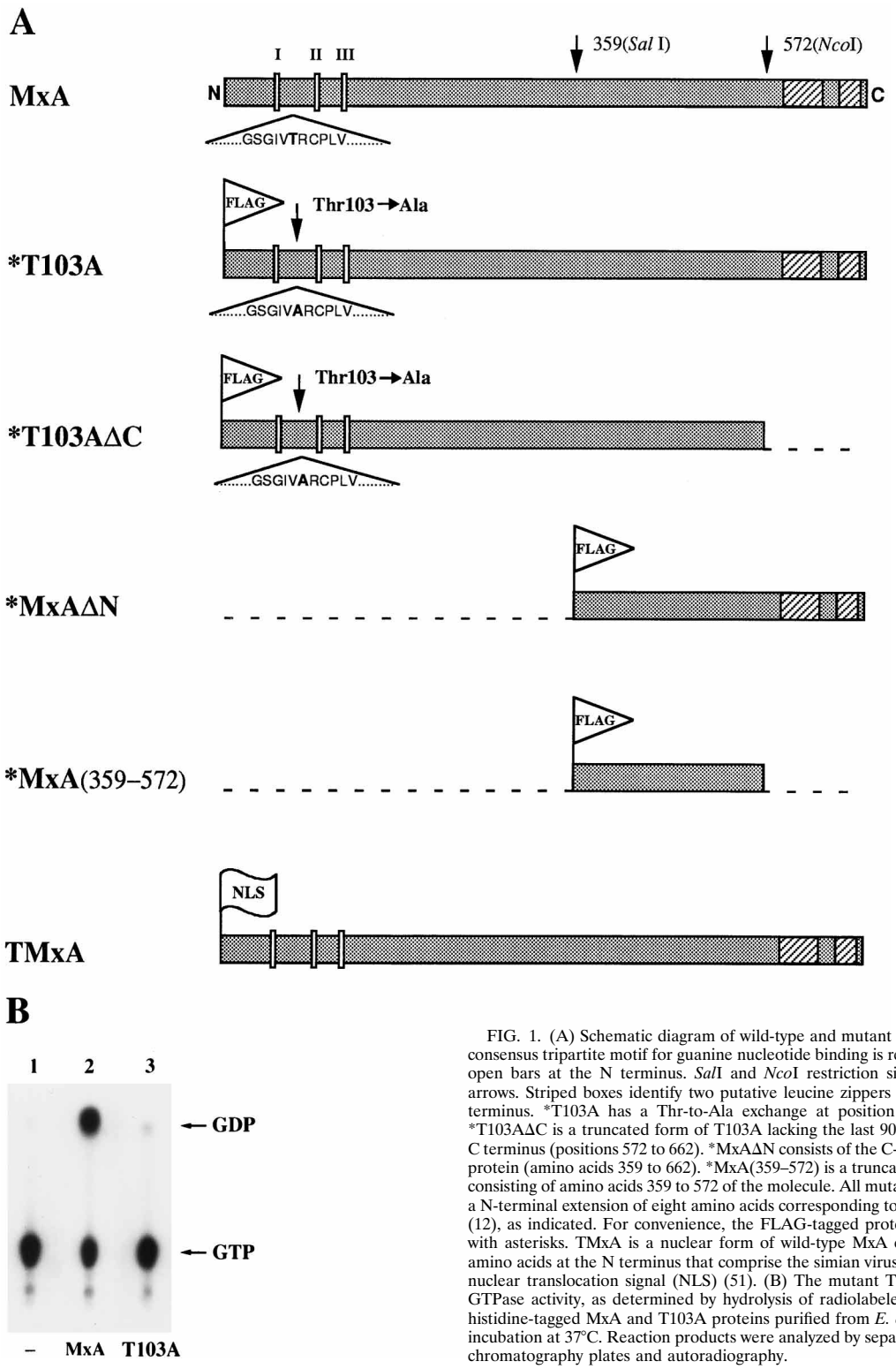

FIG. 1. (A) Schematic diagram of wild-type and mutant MxA proteins. The consensus tripartite motif for guanine nucleotide binding is represented by three open bars at the N terminus. *Sal*I and *Nco*I restriction sites are marked by arrows. Striped boxes identify two putative leucine zippers located near the C terminus. *T103A has a Thr-to-Ala exchange at position 103, as indicated. *T103AΔC is a truncated form of T103A lacking the last 90 amino acids at the C terminus (positions 572 to 662). *MxAΔN consists of the C-terminal half of the protein (amino acids 359 to 662). *MxA(359–572) is a truncated version of MxA consisting of amino acids 359 to 572 of the molecule. All mutant proteins contain a N-terminal extension of eight amino acids corresponding to the FLAG peptide (12), as indicated. For convenience, the FLAG-tagged proteins are designated with asterisks. TMxA is a nuclear form of wild-type MxA containing 18 extra amino acids at the N terminus that comprise the simian virus 40 large-T-antigen nuclear translocation signal (NLS) (51). (B) The mutant T103A protein lacks GTPase activity, as determined by hydrolysis of radiolabeled GTP to GDP of histidine-tagged MxA and T103A proteins purified from *E. coli* after 30 min of incubation at 37°C. Reaction products were analyzed by separation on thin-layer chromatography plates and autoradiography.

tagged wild-type MxA protein, indicating that the extension of eight amino acids due to the presence of the FLAG peptide at the N terminus had no negative effect on antiviral activity. Accumulation of viral antigens in cells expressing the different MxA mutants was comparable to that observed in the surrounding untransfected cells, demonstrating that each of the mutant proteins was devoid of antiviral activity.

Dominant-negative effect of mutant T103A protein. Stably transfected 3T3-MxA clones which express wild-type MxA protein constitutively and uniformly in up to a 100% of cells resist

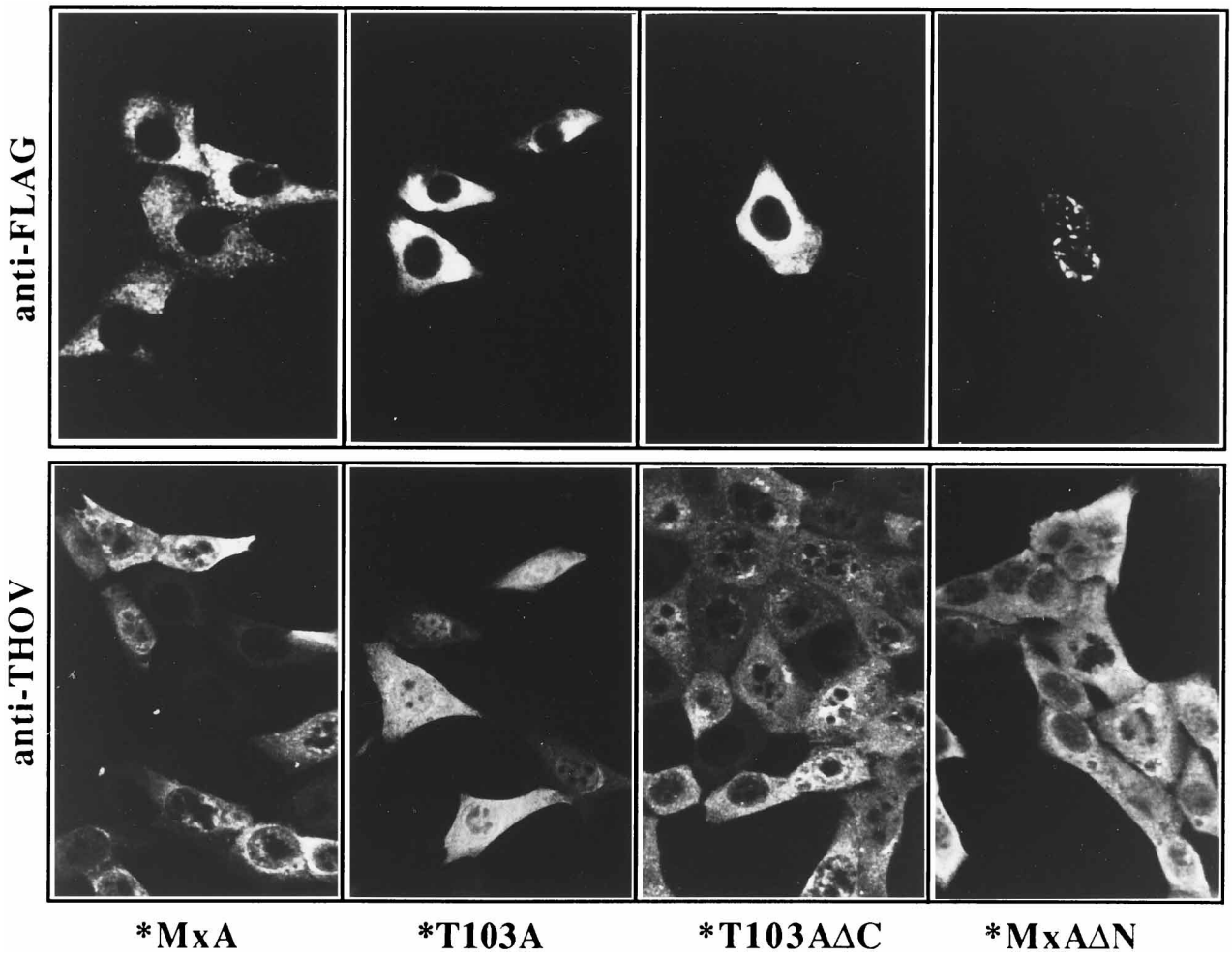

FIG. 2. Antiviral activity of wild-type and mutant MxA proteins. Transiently transfected mouse 3T3 cells expressing either FLAG-tagged wild type (*MxA) or mutant MxA proteins (*T103A, *T103AΔC, and *MxAΔN) were infected with THOV at a multiplicity of infection of 100 per cell for 16 h. Accumulation of FLAG-tagged proteins or viral proteins was analyzed by double immunofluorescence using a mixture of anti-FLAG antibody (upper panel) and a hyperimmunized guinea pig antiserum against viral antigens (lower panel).

infections with THOV (Fig. 3A), as previously shown (7). Viral antigens were not detectable in any of the MxA-expressing cells, whereas control cells showed bright staining of viral antigens. The MxA-expressing cells were therefore considered to be an ideal test system to demonstrate dominant-negative effects of mutant MxA proteins. These 3T3-MxA cells were transiently transfected with a cDNA construct expressing FLAG-tagged T103A. Figure 3A shows that 3T3 cells expressing both wild-type and mutant T103A proteins failed to mediate resistance to viral infection, indicating that the presence of T103A rendered MxA-expressing cells permissive for THOV.

The C-terminal moiety of MxA is sufficient for the dominant-negative effect. To determine which regions were critical for the observed dominant-negative effect of T103A, we expressed various truncated forms of T103A together with the full-length wild-type protein. Surprisingly, transient expression of MxAΔN (which represents only the C-terminal half of MxA [Fig. 1A]) also had a dominant-negative effect (Fig. 3A, right panel). These results indicated that sequences in the C-terminal moiety of MxA were able to neutralize the antiviral activity of the wild-type protein and were sufficient for this negative effect. To further assess the role of the C terminus, the C-terminal deletion mutant T103AΔC, lacking the last 90 amino acids (Fig. 1A), was transiently expressed in 3T3-MxA cells. Figure 3A shows that coexpression of this shortened MxA protein carrying the T103A mutation in the GTP-binding domain failed to render MxA-expressing cells permissive for virus infection. Obviously, removal of the last 90 C-terminal residues abolished the dominant-negative effect of T103A, indicating that sequences at the very C terminus were crucially involved in the capacity of T103A to neutralize the antiviral activity of MxA. However, we cannot exclude the remote possibility that the N terminus in its natural conformation contains additional regions that would be able to interfere with wild-type activity. Such domains would be hard to detect because manipulation of the C terminus seems to destroy the native conformation of the N-terminal part of MxA (39).

Virus susceptibility of MxA-expressing cells stably cotransfected with T103A. Next, the dominant-negative effect of mutant T103A was assessed by Northern blot analyses and plaque assays. To that end, a stable cell line, 3T3-MxA/T103A, coexpressing wild-type and T103A was established (see Materials and Methods). Accumulation of viral RNA was monitored 16 h after infection, using a cDNA probe specific for transcripts of the fifth segment of THOV coding for the viral nucleocapsid protein NP (Fig. 3B, upper panel). As expected, cells express-

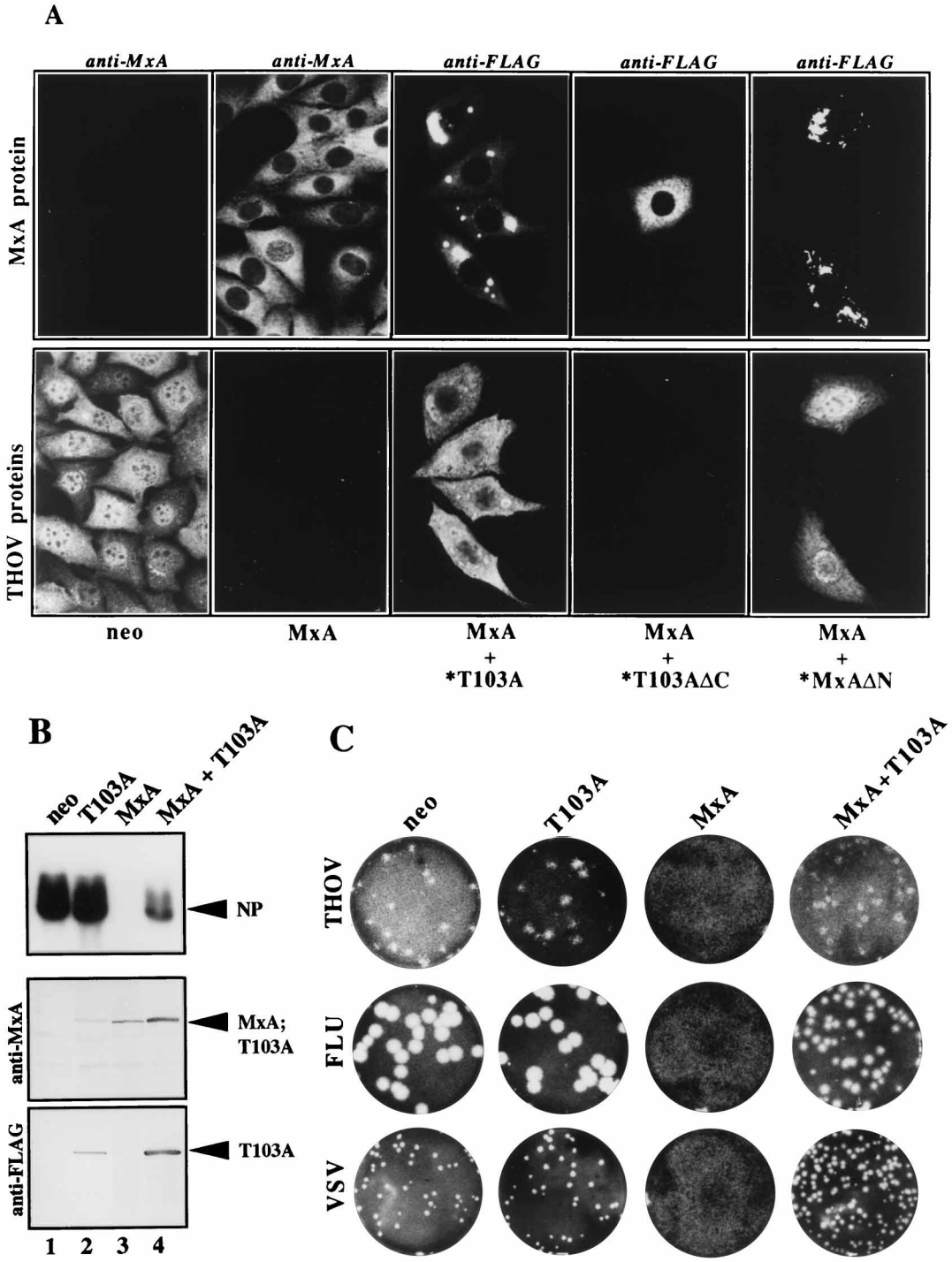

FIG. 3. Dominant-negative effect of mutant MxA proteins. (A) Detection of viral antigens in THOV-infected 3T3 cells coexpressing both wild-type and mutant MxA proteins. Monolayers consisted of stably MxA-transfected 3T3 cells expressing wild-type MxA protein alone (MxA) and stably MxA-transfected 3T3 cells transiently cotransfected to express either the FLAG-tagged mutant T103A protein (MxA + *T103A), the FLAG-tagged C-terminal deletion mutant of T103A (MxA + *T103AΔC), or the FLAG-tagged C-terminal half of MxA (MxA + MxAΔN). 3T3-SV2neo cells expressing only the neomycin resistance gene (neo) served as controls. Monolayers were infected with THOV at a multiplicity of infection of 100 per cell, and viral antigens were detected 16 h later by double-immunofluorescence staining. Wild-type MxA protein was stained with monoclonal antibody 2C12 (upper panel, anti-MxA), whereas mutant MxA proteins were stained with anti-FLAG antibody (upper panel, anti-FLAG). Viral antigens were stained simultaneously with a hyperimmunized guinea pig antiserum (lower panel). FLAG-tagged proteins are designated with asterisks. (B) Detection of *NP* transcripts of THOV by Northern blot analysis. Control 3T3-SV2neo cells expressing only the neomycin resistance gene (lane 1), stably transfected 3T3-T103A cells expressing only T103A mutant MxA protein (lane 2), 3T3-MxA cells expressing only wild-type MxA protein (lane 3), and 3T3-MxA/T103A cells coexpressing both wild-type MxA and mutant T103A protein (lane 4) were infected with THOV for 16 h. Total cellular RNA (20 μg per lane) was analyzed for accumulation of viral transcripts by Northern blotting using a radiolabeled 1.4-kb cDNA probe corresponding to the THOV *NP* gene. Expression of wild-type or mutant MxA proteins was verified by Western blotting using antibodies directed against either the MxA protein (middle panel) or the FLAG peptide (lower panel), respectively. (C) Plaque assays showing the dominant-negative effect of T103A mutant MxA protein on wild-type MxA activity against THOV, influenza A virus, and VSV. Confluent monolayers of control 3T3 cells lacking MxA (neo), 3T3-T103A cells expressing T103A mutant protein only (T103A), 3T3-MxA cells expressing wild-type MxA protein only (MxA), and 3T3-MxA/T103A cells coexpressing both wild-type MxA and T103A mutant protein (MxA + T103A) were infected with several hundred PFU of either THOV, influenza A virus strain FPV-B (FLU), or VSV. The viruses were allowed to form plaques under soft agar for 96 h (THOV), 48 h (influenza A virus), or 24 h (VSV).

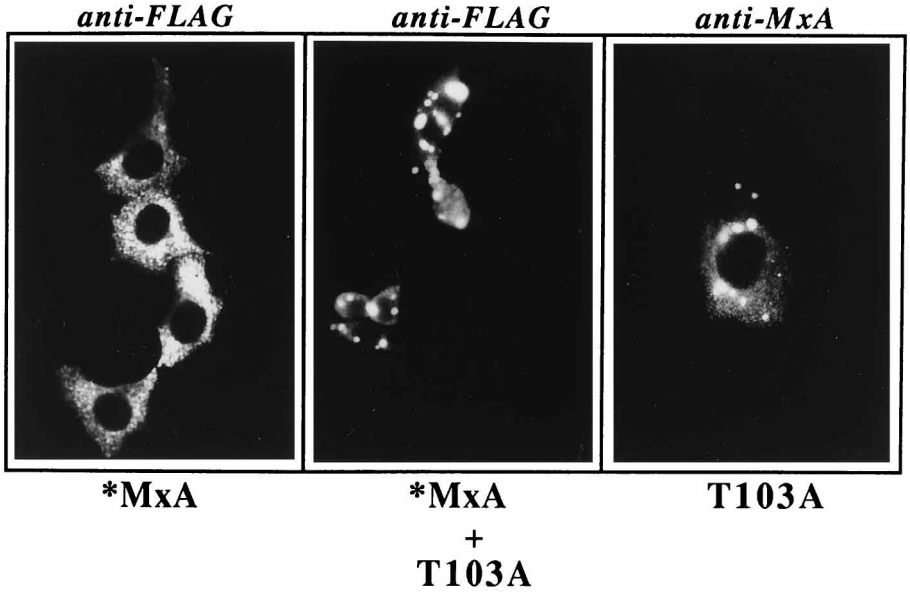

FIG. 4. Colocalization of wild-type MxA and mutant T103A protein. A first set of 3T3 cells was separately transfected either with a cDNA construct coding for wild-type MxA protein tagged with the FLAG-peptide or with a cDNA construct coding for mutant T103A. A second set of 3T3 cells was cotransfected with both constructs. The subcellular localization of the expressed proteins was analyzed by indirect immunofluorescence using either the anti-FLAG antibody to detect the wild-type protein (anti-FLAG) or a polyclonal rabbit antiserum against *E. coli*-expressed histidine-tagged MxA protein to detect T103A (anti-MxA). FLAG-tagged wild-type MxA is indicated by asterisks.

ing the mutant T103A protein were completely permissive to THOV infection, as were control cells expressing only the neomycin resistance gene (Fig. 3B, lanes 1 and 2). Cells expressing wild-type MxA protein were protected, and no viral transcripts were detectable (Fig. 3B, lane 3). However, coexpression of mutant T103A reversed the block of wild-type MxA in 3T3-MxA/T103A cells (Fig. 3B, lane 4). Viral transcripts could easily be detected, although they accumulated to lower levels than in control cells not expressing MxA (compare lane 4 with lanes 1 and 2), indicating that expression of the dominant-negative mutant did not completely overcome the antiviral activity of MxA. Expression of wild-type or mutant proteins was measured by Western blotting using antibodies directed either against MxA protein (Fig. 3B, middle panel) or the FLAG peptide (Fig. 3B, lower panel), respectively. Next, we examined whether THOV was able to form infectious virus progeny in these cells. Figure 3C shows that coexpression of mutant T103A enabled the virus to multiply and form plaques, comparable to the situation in permissive control cells. In summary, these data show that the dominant-negative effect of T103A can be demonstrated at different levels of the virus multiplication cycle.

The dominant-negative effect includes several MxA-sensitive viruses. The next question was whether the dominant-negative effect could also be demonstrated with MxA-sensitive viruses other than THOV. Influenza A virus and VSV are both susceptible to inhibition by wild-type MxA in different cell lines (7, 31). In monolayers of 3T3 cells expressing mutant T103A protein only, both viruses formed distinct plaques similar in size to those found in control cells expressing only the neomycin resistance gene (Fig. 3C). In MxA-expressing cells, no plaques were visible. Again, coexpression of T103A suppressed wild-type MxA activity (Fig. 3C), demonstrating that the dominant-negative effect of T103A is not restricted to THOV but applies to all three viruses tested.

Dominant-negative and neutral mutants interact directly with wild-type MxA. To look for a direct interaction between the dominant-negative mutant T103A and the wild-type MxA protein, we analyzed the subcellular distribution of coexpressed proteins. Wild-type protein has a diffuse granular distribution, whereas the dominant-negative T103A mutant forms large aggregates (Fig. 4). When coexpressed with T103A, FLAG-tagged wild-type MxA (Fig. 4, left panel) changed its normal behavior and accumulated in large aggregates (Fig. 4, middle panel), as did T103A alone (Fig. 4, right panel). These data suggested that wild-type MxA and the dominant-negative mutant are in close contact when expressed in the same cell.

To corroborate these findings, we devised a second experiment using a stringent test for tight association of partner molecules in living cells. We took advantage of the availability of TMxA, a nuclear form of wild-type MxA that accumulates in the cell nucleus (Fig. 5A, lower left panel) due to a foreign nuclear translocation signal at its N terminus (Fig. 1) (51). We reasoned that a tight association of MxA mutants with TMxA should result in translocation of mutant proteins from the cytoplasm into the nucleus. Figure 5A shows the intracellular localization of the various cytoplasmic FLAG-tagged MxA mutants when expressed alone (upper panel) or together with TMxA (lower panel). All MxA mutants were translocated into the nucleus when expressed together with TMxA. Interestingly, MxAΔN, the C-terminal half of MxA, was moved to the nucleus with TMxA in the same way as the C-terminal deletion mutant, T103AΔC. Both mutant proteins have a region encoded by sequences between the *Sal*I and *Nco*I restriction sites. It was therefore conceivable that this region (amino acids 359 to 572) contains an interaction domain which is important for the association of both proteins with TMxA. To prove this, we generated an additional MxA deletion mutant corresponding to positions 359 to 572 of the wild-type MxA protein, called MxA(359–572). Figure 5B shows the intracellular localization of FLAG-tagged MxA(359–572) when expressed together with wild-type MxA (left panel) or together with TMxA (right panel). MxA(359–572) formed heterooligomers with TMxA and was translocated to the nucleus, demonstrating that the region

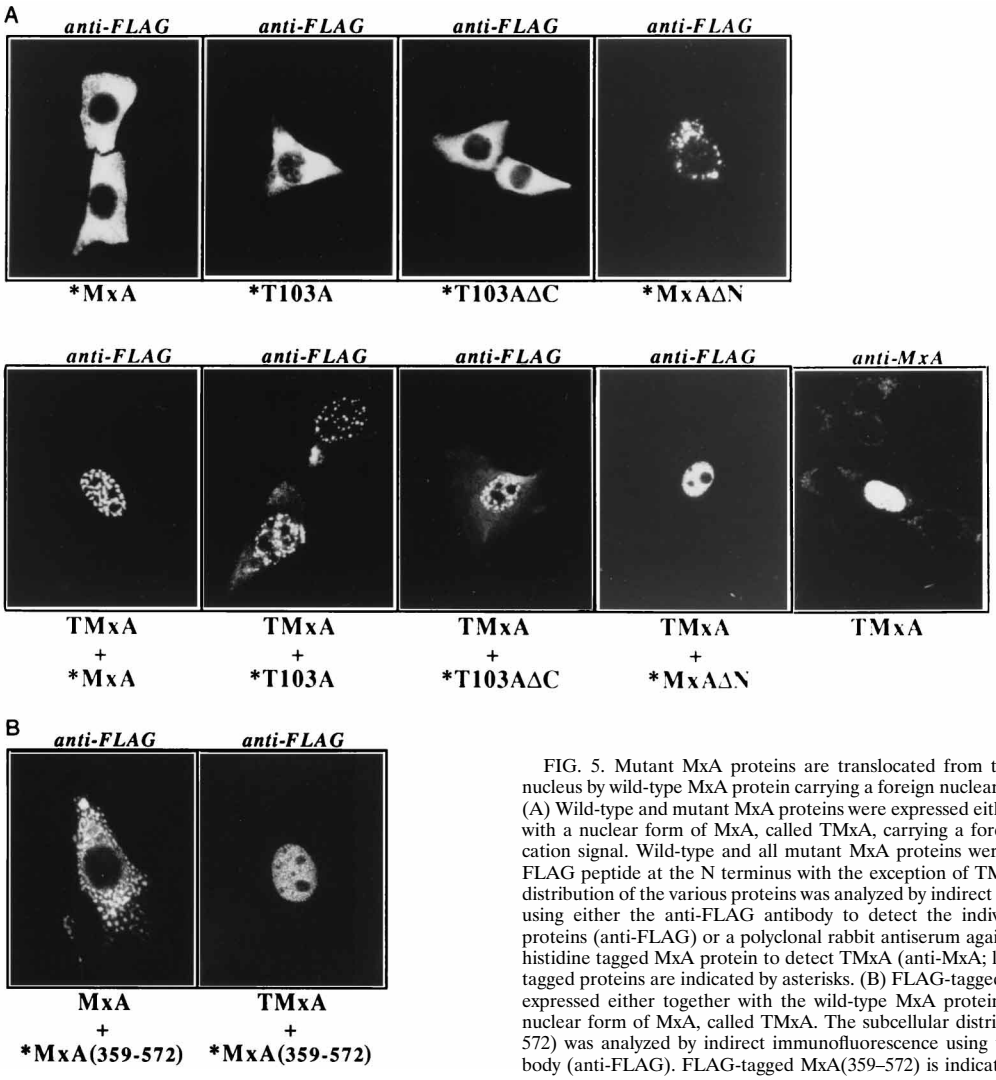

FIG. 5. Mutant MxA proteins are translocated from the cytoplasm to the nucleus by wild-type MxA protein carrying a foreign nuclear translocation signal. (A) Wild-type and mutant MxA proteins were expressed either alone or together with a nuclear form of MxA, called TMxA, carrying a foreign nuclear translocation signal. Wild-type and all mutant MxA proteins were equipped with the FLAG peptide at the N terminus with the exception of TMxA. The subcellular distribution of the various proteins was analyzed by indirect immunofluorescence using either the anti-FLAG antibody to detect the individual FLAG-tagged proteins (anti-FLAG) or a polyclonal rabbit antiserum against *E. coli*-expressed histidine tagged MxA protein to detect TMxA (anti-MxA; lower panel). FLAG-tagged proteins are indicated by asterisks. (B) FLAG-tagged MxA(359–572) was expressed either together with the wild-type MxA protein or together with a nuclear form of MxA, called TMxA. The subcellular distribution of MxA(359–572) was analyzed by indirect immunofluorescence using the anti-FLAG antibody (anti-FLAG). FLAG-tagged MxA(359–572) is indicated by an asterisk.

between amino acids 359 and 572 contains indeed a domain important for intermolecular MxA-MxA-interactions.

DISCUSSION

The main conclusion from the experiments presented here is that mutant MxA proteins devoid of both GTPase and antiviral activities can interfere with the antiviral function of the wild-type MxA protein in vivo and that the C-terminal moiety is necessary and sufficient for this effect. The present findings are consistent with previous reports on mutant forms of dynamin and VPS1 showing that mutations which affect GTP binding exhibit a dominant-interfering effect on wild-type dynamin and VPS1 activities. Moreover, expression of only a C-terminal fragment of dynamin or VPS1 exerts the same dominant-negative effect, whereas C-terminal deletion mutants are neutral, having lost interfering activity (48, 49).

Mechanistically, there are several ways to explain the dominant-negative effect of the mutant proteins used in this study. A likely explanation is the formation of inactive hetero-oligomers consisting of mutant and wild-type MxA proteins. Purified MxA proteins are known to form high-molecular-weight oligomers (24, 34). The fine granules observed by immunofluorescence staining of wild-type MxA (42) most likely represent MxA aggregates in the cytoplasm of cells where they accumulate and may associate with cytoskeletal or vesicular structures (13). We do not know whether a monomeric or oligomeric structure is the functional entity of MxA and whether additional cellular proteins participate in the formation of MxA oligomers. In either case, hetero-oligomerization with dominant-negative MxA mutants may be deleterious if nonfunctional hetero-oligomers are formed or cells are depleted from functional monomers. It should be stressed here that physical interaction between mutant and wild-type MxA proteins was not sufficient to establish a dominant-negative effect. The neutral mutants were also able to form hetero-oligomers with wild-type MxA, as demonstrated by their cotranslocation together with TMxA into the cell nucleus. Hence, additional properties are seemingly required for the manifestation of the interfering effect. Wild-type MxA homo-oligomers bind and hydrolyze GTP (34, 39). It remains to be determined whether hetero-oligomerization with T103A or MxAΔN suppresses the capacity of the wild-type protein to bind and hydrolyze GTP and whether the neutral mutant T103AΔC has a different effect. It has previously been shown that accumulation in different subcellular compartments may determine antiviral activity

and specificity (17, 52). Here, dominant-negative MxA mutants were shown to exhibit an abnormal granular cytoplasmic distribution, whereas the immunofluorescence staining pattern of the neutral C-terminal deletion mutant was indistinguishable from that of wild-type MxA. In view of the unusual subcellular localization of T103A and MxAΔN, an intriguing possibility is that these mutant proteins are missorted and, by binding to MxA, may sequester the wild-type protein away from its normal cellular location where it would otherwise be able to block virus multiplication.

An alternative interpretation of our results is that the interfering mutant proteins compete with wild type for a common viral or cellular partner, an activity that would be expected to involve the C terminus. Unfortunately, little is known about candidate partner molecules. Functional tests with influenza A virus and VSV pointed to components of the viral polymerase complex as targets (14, 21, 40, 45), but biochemical data demonstrating physical interactions are missing. Actin and tubulin have been reported to be associated with MxA (13), and circumstantial evidence implicates additional host cell factors that need to be identified (17, 36, 38).

The present findings highlight the importance of the C terminus for antiviral activity and complement previous work. Leucine repeats that are conserved in all Mx proteins (24, 23) and form two putative leucine zippers have been identified near the C terminus of MxA (Fig. 1). It has previously been shown that the C-terminal region determines antiviral specificity because a single amino acid substitution replacing the glutamic acid at position 645 by arginine within the distal leucine zipper changes the antiviral properties of MxA in such a way that the mutant has lost activity against VSV while retaining activity against influenza virus and THOV (7, 51). This observation suggests a direct interaction between MxA, in particular its C-terminal end, with viral target structures (44), although other scenarios implicating cellular partner molecules are also possible (17). It is conceivable that leucine repeats forming an amphipathic helix are involved in cellular and viral target recognition. Accordingly, substituting leucine residues in the distal leucine zipper for amino acids that presumably abrogate the amphipathic character of the helix resulted in loss of antiviral activity of mouse Mx1 protein (8). Similar mutations in either the proximal or the distal leucine zipper motif abolished antiviral activity of human MxA protein (28), and mutations in the proximal leucine zipper reportedly also abolished the interfering capacity of dominant-negative MxA mutants (4), indicating that this proximal leucine-rich region is instrumental in mediating the dominant-negative effect.

Finally, our results demonstrate that MxA molecules interact tightly with each other in the living cell and that a region of 213 amino acids (positions 359 to 572) is responsible for this intermolecular interaction. To demonstrate this, we took advantage of the properties of TMxA, a modified form of wild-type MxA that accumulates in the cell nucleus (51). We could clearly show that the cytoplasmic form of wild-type MxA was translocated together with the nuclear form of TMxA into the nucleus, where both forms accumulated. This elegant test system can now be used to better define the domains required for MxA-MxA interactions in vivo. Two different Mx domains have previously been proposed to be responsible for oligomerization: the putative leucine zipper repeats near the carboxyl end (23, 24) and a self-assembly motif originally identified close to the first GTP-binding consensus sequence in the N-terminal moiety of mouse Mx1 protein (26). However, in case of human MxA protein, experimental evidence for involvement in oligomerization of one or the other of these separate domains is lacking. We show here that MxAΔN, the C-terminal half of MxA, was moved to the nucleus together with TMxA in the same way as full-length MxA. Likewise, the C-terminal deletion mutant, T103AΔC, accumulated in the nucleus when coexpressed with TMxA. Both mutant MxA proteins have a region encoded by sequences between the *Sal*I and *Nco*I restriction sites containing the interaction domain (amino acids 359 to 572) which is shown here to be responsible for the tight association with wild-type MxA (Fig. 5B). In summary, our data demonstrate that wild-type MxA forms oligomers with modified and mutant proteins of MxA in vivo. A single amino acid substitution in the GTP-binding domain resulted in a GTPase-defective and antivirally inactive mutant which exhibited a dominant-interfering effect on the antiviral activity of wild-type MxA. An intact C terminus was required for interference. Moreover, expression of the C-terminal moiety exerted the same dominant-negative effect, indicating that sequences in the C-terminal half of MxA are important in mediating effector function.

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