

## Antiviral Determinants of Rat Mx GTPases Map to the Carboxy-Terminal Half

LUDGER JOHANNES,<sup>†</sup> RAVI KAMBADUR,<sup>‡</sup> HELEN LEE-HELLMICH,<sup>§</sup>  
COLIN A. HODGKINSON,<sup>||</sup> HEINZ ARNHEITER, AND ELLEN MEIER\*

*Laboratory of Developmental Neurogenetics, National Institute of  
Neurological Disorders and Stroke, Bethesda, Maryland 20892*

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**Rat Mx2 and rat Mx3 are two alpha/beta interferon-inducible cytoplasmic GTPases that differ in three residues in the amino-terminal third, which also contains the tripartite GTP-binding domain, and that differ in five residues in the carboxy-terminal quarter, which also contains a dimerization domain. While Mx2 is active against vesicular stomatitis virus (VSV), Mx3 lacks antiviral activity. We mapped the functional difference between Mx2 and Mx3 protein to two critical residues in the carboxy-terminal parts of the molecules. An exchange of either residue 588 or 630 of Mx2 with the corresponding residues of Mx3 abolished anti-VSV activity, and the introduction of the two Mx2 residues on an Mx3 background partially restored anti-VSV activity. These results are consistent with the facts that Mx2 and Mx3 have similar intrinsic GTPase activities and that the GTPase domain of Mx3 can fully substitute for the GTPase domain of Mx2. Nevertheless, the amino-terminal third containing the GTP-binding domain is necessary for antiviral activity, since an amino-terminally truncated Mx2 protein is devoid of anti-VSV activity. Furthermore, Fab fragments of a monoclonal antibody known to neutralize antiviral activity block GTPase activity by binding an epitope in the carboxy-terminal half of Mx2 or Mx3 protein. The results are consistent with a two-domain model in which both the conserved amino-terminal half and the less-well-conserved carboxy-terminal half of Mx proteins carry functionally important domains.**

Mx proteins are large, alpha/beta interferon-inducible GTPases that are found in vertebrates from fish to humans and are named after the prototype Mx protein, murine Mx1 (5, 12, 20, 26). Murine Mx1 protein is expressed in mice after infection with viruses and accumulates in the nuclei of cells in and around the anatomical foci, where the viruses replicate (6). In these cells, Mx1 protein specifically interferes with the replication and spread of influenza viruses and Thogoto virus (6, 11). Mice with mutations in the gene coding for Mx1 succumb to infection with these viruses, since alpha/beta interferon alone, without help from Mx1, is insufficient for protection (30) [for a review, see reference 5]).

Most mammals, including humans, possess at least two alpha/beta interferon-inducible Mx genes. The majority of these genes encode cytoplasmic proteins that may have distinct antiviral specificities (1, 5, 7, 8, 13, 15). For example, human MxA protein inhibits vesicular stomatitis virus (VSV) transcription (28) as well as a posttranscriptional step in the influenza virus replication cycle (22, 23). Rat Mx2 (rMx2) protein interferes only with VSV but not influenza virus (16), even though it can be tricked into inhibiting influenza virus when engineered to

move into the cell nucleus (14). rMx3 protein is inactive against either influenza virus or VSV (16).

Targeted mutagenesis has shown that GTPase activity is required for the antiviral activity of Mx proteins (10, 19, 24). However, the amino-terminal domain which contains the GTP-binding domain is not sufficient for antiviral activity, since molecules lacking the carboxy-terminal domain or displaying point mutations, deletions, or linker-insertion mutations in the carboxy-terminal domain are also devoid of antiviral activity. In some of these carboxy-terminal mutants, GTPase activity is also affected, but in others, it remains intact (10, 19, 27, 28, 31). These mutational analyses suggest a two domain-model with intra- or intermolecularly interacting domains. In fact, aggregates of amino- and carboxy-terminal proteolytic fragments of human MxA protein have GTPase activity, in contrast to the corresponding isolated fragments which lack such activity (27). Nevertheless, it is important to recognize that the mutational analyses are largely based on loss-of-function mutations, thus rendering a precise delineation of antiviral domains difficult. An exception may be the carboxy-terminal point mutation in human MxA protein, E645→R, that resulted in a protein that selectively lost anti-VSV activity but retained anti-influenza virus and GTPase activities (31).

In order to identify which domains are responsible for the functional difference between the rMx2 and rMx3 proteins, we have determined the intrinsic GTPase activities of the two proteins, mapped an epitope recognized by a neutralizing monoclonal antibody, and assayed the antiviral activities of a series of reciprocal chimeric molecules expressed in Mx-negative fibroblasts.

GTPase activities were determined with recombinant proteins purified from *Escherichia coli*. The two proteins were each expressed as fusion proteins with the maltose binding protein, partially purified by maltose affinity chromatography, cleaved with factor Xa to remove the maltose binding moiety,

\* Corresponding author. Present address: Center for NeuroVirology and NeuroOncology, Allegheny University for the Health Sciences, 245 N. Fifteenth St., Mail Stop 406, Philadelphia, PA 19102. Phone: (215) 762-3525. Fax: (215) 762-8328. E-mail: meiere@auhs.edu.

<sup>†</sup> Present address: Institut Curie, CNRS UMR 144, Compartimentation et Dynamique Cellulaire, F-75005 Paris, France.

<sup>‡</sup> Present address: AgResearch, Hamilton, New Zealand.

<sup>§</sup> Present address: Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555.

<sup>||</sup> Present address: Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109-0650.

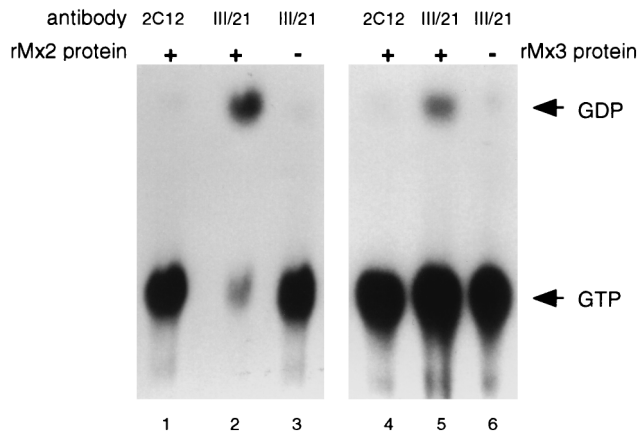


FIG. 1. GTPase activity of rMx2 and rMx3 proteins. Samples (125 ng) of MonoQ- and Superose-12-purified rMx2 and rMx3 proteins were left untreated (lanes 1, 2, 4, and 5) or were heat treated (lanes 3 and 6). The samples were then incubated for 60 min with radiolabeled GTP in the presence of 1 mg of FPLC-purified Fab fragments of monoclonal antibody 2C12 (lanes 1 and 4) or III/21 (lanes 2, 3, 5, and 6) per ml. The conversion of GTP to GDP was assessed by chromatography of polyethyleneimine-cellulose plates followed by autoradiography.

and further purified by fast protein liquid chromatography (FPLC) with MonoQ and Superose-12 columns. rMx2 and rMx3 proteins hydrolyzed [ $\alpha$ - $^{32}$ P]GTP with  $K_m$  values of 130 and 150  $\mu$ M, specific GTPase activities of 100 and 105 nmol/min/mg, and turnover numbers of 7.9 and 7.5  $\text{min}^{-1}$ , respectively. These values are in the range of those obtained for human MxA and murine Mx1 (12, 19–21, 26 [for a review, see reference 5]). FPLC-purified Fab fragments of a monoclonal antibody, 2C12, known to neutralize the antiviral activities of both murine and rat Mx1 proteins (2, 9, 29) and to cross-react with all three rMx proteins (15, 16), completely inhibited the GTPase activity of recombinant Mx2 and Mx3 proteins; similarly, purified Fabs derived from an unrelated monoclonal antibody (4) had no effect (Fig. 1). These results demonstrate that the GTPase activities were not generated by potential contaminants and suggest that the differences between the rMx2 and rMx3 proteins in antiviral activity *in vivo* were unlikely due to differences in their GTPase activities.

The fact that monoclonal Fabs neutralized GTPase activity prompted efforts to determine the corresponding epitope. To this end, different 3'-terminally truncated RNAs were *in vitro* transcribed and translated from an rMx3 cDNA, and the truncated protein products consisting of the amino-terminal 280, 430, and 469 amino acids were analyzed by immunoprecipitation. Antibody 2C12 precipitated full-length rMx3 and rMx3 (1–469) but not rMx3(1–280) and rMx3(1–430) (Fig. 2). These data suggest that the 2C12-binding site is located between residues 430 and 469 of rMx3 and, because of sequence identity in this region, also those of rMx2. Thus, 2C12 appears to neutralize GTPase activity, not by binding directly to the GTPase domain, but indirectly, by binding to a domain in the carboxy-terminal half.

To determine which sequence differences were responsible for the difference in anti-VSV activity, we generated a series of reciprocal chimeras between rMx2 and rMx3 proteins, expressed them in Mx-negative mouse 3T3 cells (30), and assayed them for level of accumulation, intracellular distribution, and antiviral activity. The results are shown in Fig. 3. All chimeric proteins schematically depicted in Fig. 3A accumulated to similar levels when assayed by immunofluorescence (data not

shown) or Western blotting (Fig. 3D). Interestingly, all chimeric proteins with an rMx2-type amino terminus migrated like parental rMx2 ( $M_r = 75,000$ ) protein, and all chimeric proteins with an rMx3-type amino terminus migrated like parental rMx3 protein ( $M_r = 77,000$ ). This indicated that the previously recognized migration difference between rMx2 and rMx3 is due to the sequence differences in the amino terminus. Immunolabeling showed that the parental proteins and their chimeric derivatives were all strictly cytoplasmic; however, with respect to their staining patterns, they fell into two groups: staining was either granular, similar to that of the parental rMx2 protein, or diffuse, similar to that of the parental rMx3 protein, and this difference correlated well with the antiviral activities of the individual chimeras (Fig. 3C) (14, 16).

To determine the antiviral activities of the chimeric molecules, we injected the expression plasmids into the nuclei of 3T3 cells, infected the cells 24 h later at high multiplicity with VSV, and then double stained them for expression of Mx and viral proteins. Similar to previous results (14, 16), 2% of cells expressing rMx2 protein and 88% of cells expressing rMx3 protein were positive for VSV (Fig. 3B). Chimeric rMx2/3 protein lacked anti-VSV activity, similar to rMx3 protein (38 of 41 [93%] cells VSV positive), while the reciprocal chimeric rMx3/2 protein retained considerable anti-VSV activity (6 of 46 [13%] cells VSV positive), thus resembling rMx2 protein (Fig. 3B). These results indicate that the sequence differences in the carboxy-terminal parts, but not those in the amino-terminal parts, must be responsible for the lack of antiviral activity of rMx3 protein.

To test the roles of the sequence differences in the carboxy-terminal domains, reciprocal site-specific mutations were generated individually for positions 518, 561, 564, 588, and 630. As shown in Fig. 3B, the chimeric proteins rMx2(S518A), rMx2(S561A), and rMx2(K564P) were as active as the parental rMx2 protein, with the numbers and percentages of VSV-positive cells per Mx protein-expressing cell being 2 of 39 (5%), 1 of 40 (3%), and 8 of 80 (10%) cells, respectively. In

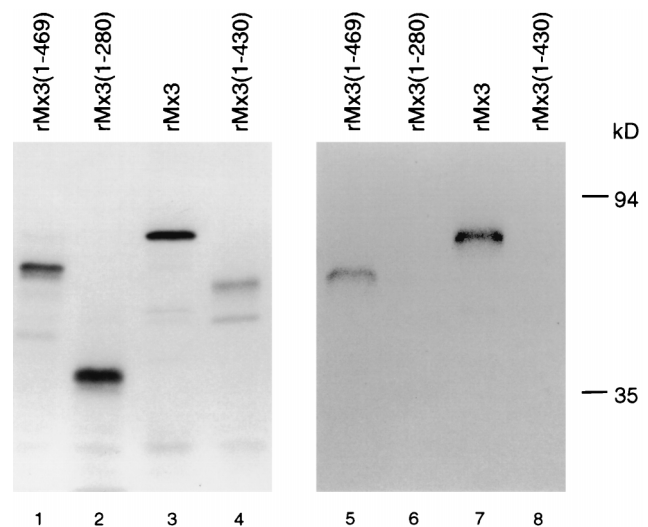


FIG. 2. Mapping of the 2C12-binding site. Plasmid pMx223, which contains an rMx3 cDNA downstream of the SP6 promoter, was cut at four different positions corresponding to amino acid positions 280, 430, 469, and 659 (full length). Capped runoff transcripts were synthesized with SP6 polymerase and *in vitro* translated in a rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine. The labeled products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before (lanes 1 to 4) and after (lanes 5 to 8) immunoprecipitation with antibody 2C12. The proteins were visualized by fluorography.

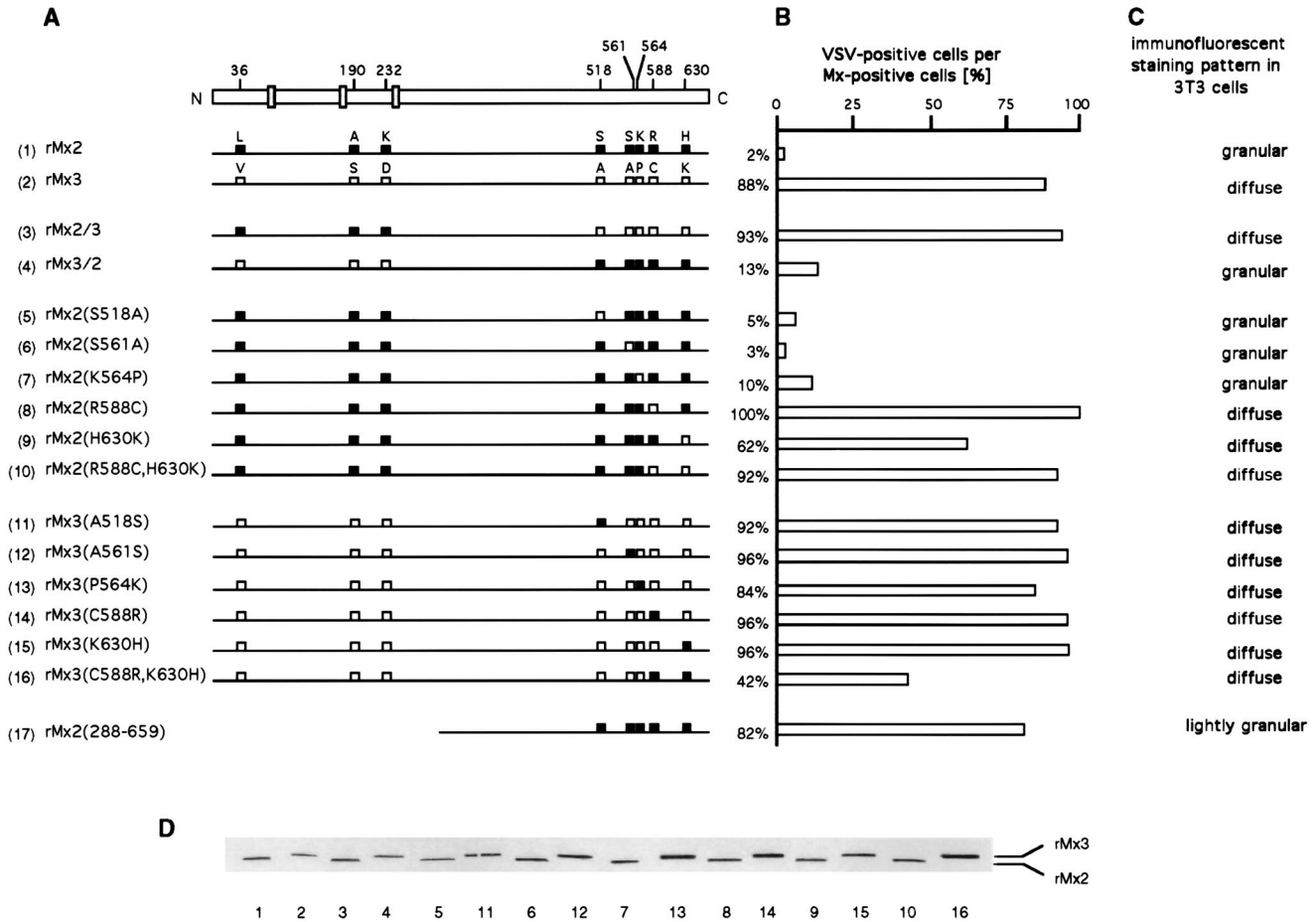


FIG. 3. Antiviral activity, immunofluorescent staining pattern, and apparent molecular weight of mutant and wild-type rMx2 and rMx3 proteins in BALB/3T3 cells. (A) Linear protein maps. The numbers at the top refer to the amino acid positions where wild-type rMx2 and rMx3 proteins differ from each other, and the letters refer to the particular amino acids (single-letter code) found at these positions. The solid and open boxes indicate amino acids specific for the rMx2 and rMx3 proteins, respectively. The shaded boxes show the tripartite GTP-binding domain. (B) Anti-VSV activity. Plasmids encoding mutant or wild-type rMx2 and rMx3 proteins were injected into the nuclei of Mx1-negative mouse BALB/3T3 cells. Eighteen hours later, the cells were infected with VSV, fixed, and double labeled for Mx and VSV proteins. Anti-VSV activity is given as the percentage of cells double labeled for VSV and Mx relative to the total number of Mx-positive cells. Each bar represents the combined results obtained from three dishes in each of three independent experiments. Of uninjected control cells, 99% were VSV positive. (C) Immunofluorescent staining pattern. Mouse BALB/3T3 cells were injected as described above, incubated for 18 h, and then fixed and stained for Mx protein with the monoclonal antibody 2C12. (D) Apparent molecular weights. BALB/3T3 cells were transfected with rMx expression plasmids. Total cell extracts were prepared 40 h later and subjected to Western blot analysis with a polyclonal antibody raised against rMx3 protein. The numbers at the bottom refer to the Mx proteins as indicated in panel A.

contrast, rMx2(R588C) completely lacked anti-VSV activity (48 of 48 [100%] cells VSV positive) and rMx2(H630K) had significantly reduced anti-VSV activity (33 of 53 [63%] cells VSV positive). Thus, the replacement of amino acids at either positions 588 or 630 can separately impart susceptibility to VSV. However, the analysis of the reciprocal chimeras showed that none of the single-amino-acid substitutions was able to render rMx3 antivirally active. As shown in Fig. 3B, the numbers and percentages of VSV-positive cells relative to the numbers of Mx protein-expressing cells were 48 of 52 (92%) for rMx3(A518S), 51 of 53 (96%) for rMx3(A561S), 36 of 43 (84%) for rMx3(P564K), 50 of 51 (98%) for rMx3(C588R), and 52 of 54 (96%) for rMx3(K630H). Thus, neither an arginine at position 588 nor a histidine at position 630 restored anti-VSV activity in Mx3 protein. However, the simultaneous introduction of both of these amino acids into rMx3 protein had an effect. rMx3(C588R, K630H) acquired activity, although not as full as that of wild-type rMx2 (36 of 86 cells [42%] VSV positive) (Fig. 3B). This suggested that amino acids other than the essential arginine at position 588 and the

essential histidine at position 630 also contributed to the anti-VSV activity of rMx2 protein.

Although the results presented above demonstrated that the differences in the carboxy-terminal parts of the molecules are responsible for the differences in their antiviral activities, the amino-terminal third with its GTPase domain is still important for activity. Figure 3B shows that an amino-terminally truncated rMx2 protein, rMx2(299–659), that consisted of the carboxy-terminal 471 residues lacked anti-VSV activity (40 of 49 [82%] cells VSV positive). These results are consistent with recent findings for the human MxA protein (25).

In summary, our results show that amino-terminal truncation of rMx2 protein destroys its antiviral activity, but that the amino-terminus of rMx3, itself an antivirally dead molecule, can confer antiviral activity upon the carboxy-terminal half of rMx2 protein. Furthermore, an antibody recognizing an epitope in the carboxy-terminal half neutralizes both antiviral and GTPase activity. These observations support a two-domain model of Mx proteins whereby the amino-terminal portion provides an essential GTP binding function and the carboxy-



terminal portion provides domains that regulate GTP binding. Our observations are consistent with the results of a number of studies of related Mx proteins (10, 17, 24, 25, 27, 28). For instance, the fact that an antibody modulates GTPase activity by binding in the carboxy-terminal half is supported by the recent observation that an internal deletion mutant of human MxA lacking the sequences comprising this epitope displayed greatly reduced GTPase activity (27). Also, the mapping of critical residues to positions 588 and 630 of rMx2 or rMx3 proteins is interesting in light of the fact that upon sequence alignment, residue 630 maps close to residue 645 of human MxA, which is critical for the antiviral specificity of this Mx protein (31), and both residues 588 and 630 are in close proximity to the leucine zipper region, which is required for antiviral activity of mouse Mx1 protein (18). These observations clearly establish that both the conserved amino-terminal portion and the less-well-conserved carboxy-terminal portion carry important functional domains and that these domains interact with each other. It remains to be established, however, whether these interactions are intramolecular or intermolecular.

Our finding that antibody 2C12 blocks GTPase activity may explain the findings of previous microinjection experiments (5). This antibody, when introduced into the cytoplasm of rat cells that were subsequently treated with alpha/beta interferon to induce Mx proteins, rendered these cells susceptible to influenza virus infection (2). However, the cells still became resistant to VSV, although, based on our present results, rMx2 protein should have been neutralized as well. This apparently paradoxical observation can be explained if we assume that interferon-treated rat cells are protected against VSV even if rMx2 were nonfunctional. This interpretation is likely, since interferon-treated mouse cells that lack functional Mx proteins entirely are fully protected against VSV (3, 5).

An explanation for how the different domains of Mx proteins exert their antiviral functions may eventually come from a determination of their three-dimensional structure and the analysis of cellular and/or viral target molecules they interact with. Interestingly, all naturally occurring mutations in mouse Mx1 protein that render it nonfunctional occur in the carboxy-terminal half. This may be pure coincidence, but it is also possible that amino-terminal mutations that affect GTPase activity would generate molecules with dominant-negative functions, as was recently demonstrated for human MxA (25), and that such dominant-negative molecules would not be tolerated. However, intolerance towards dominant-negative Mx proteins could hardly be explained if they simply competed with wild-type Mx for a viral target; both interferon and Mx can be induced by viruses that are not subject to inhibition by Mx, and the lack of functional Mx is compatible with life, at least for mice. Rather, the danger of expressing dominant negative molecules would have to result from inappropriate interaction of the dominant negative molecules with a host protein. Along the same line, we would predict that rMx3 protein, although being antivirally inactive, is not an efficient dominant-negative molecule, since it is naturally synthesized during each interferon stimulation.

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