# Mx Proteins: Antiviral Gatekeepers That Restrain the Uninvited

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## SUMMARY

Fifty years after the discovery of the mouse *Mx1* gene, researchers are still trying to understand the molecular details of the antiviral mechanisms mediated by Mx proteins. Mx proteins are evolutionarily conserved dynamin-like large GTPases, and GTPase activity is required for their antiviral activity. The expression of *Mx* genes is controlled by type I and type III interferons. A phylogenetic analysis revealed that *Mx* genes are present in almost all vertebrates, usually in one to three copies. Mx proteins are best known for inhibiting negative-stranded RNA viruses, but they also inhibit other virus families. Recent structural analyses provide hints about the antiviral mechanisms of Mx proteins, but it is not known how they can suppress such a wide variety of viruses lacking an obvious common molecular pattern. Perhaps they interact with a (partially) symmetrical invading oligomeric structure, such as a viral ribonucleoprotein complex. Such an interaction may be of a fairly low affinity, in line with the broad target specificity of Mx proteins, yet it would be strong enough to instigate Mx oligomerization and ring assembly. Such a model is compatible with the broad "substrate" specificity of Mx proteins: depending on the size of the invading viral ribonucleoprotein complexes that need to be wrapped, the assembly process would consume the necessary amount of Mx precursor molecules. These Mx ring structures might then act as energy-consuming wrenches to disassemble the viral target structure.

## INTRODUCTION

The interferon (IFN) system is the first line of defense against animal viruses. Binding of type I or III IFNs to their receptors (IFNAR1/2 and IL-28Rα/IL-10Rβ, respectively) induces an anti-viral state within the cell by inducing the transcription of many IFN-stimulated genes. The antiviral activities of some of these genes are well understood (reviewed in reference 1), with one of the best studied being *Mx1*.

*Mx* genes exist in nearly all vertebrate genomes, from fish to primates, and they are active mainly against RNA viruses (Table 1). Mx proteins from different species possess distinct antiviral activities, and the subcellular localization of an Mx protein contributes to its antiviral specificity. In general, nuclear Mx proteins (e.g., mouse Mx1) protect against viruses that replicate in the nucleus, such as influenza virus and Thogoto virus (THOV) (2–10), whereas cytoplasmic forms (e.g., mouse Mx2) inhibit replication of vesicular stomatitis virus (VSV) and other viruses that replicate in the cytoplasm (11–13). The human MxA protein is cytoplasmic and has a broad antiviral spectrum that is seemingly unrelated to the intracellular replication site of the virus (5, 14–30). Antiviral activity has also been described for fish Mx proteins (31–37). For example, Atlantic salmon encodes multiple Mx proteins, all of which can suppress infectious salmon anemia virus (ISAV), a fish orthomyxovirus (33, 38).

The discovery of the *Mx1* gene was reported 50 years ago by Lindenmann et al. (3, 39) and was based on the resistance of an inbred mouse strain to influenza virus infection. Unlike most other mouse strains, A2G mice are highly resistant to influenza

TABLE 1 Antiviral spectrum of Mx proteins

| Mx protein(s) | Localization | Antiviral activity | Virus family | Genome | Reference(s) |
|---|---|---|---|---|---|
| Mouse Mx1 | Nucleus | Influenza virus | Orthomyxoviridae | ssRNA (−) | 2–6, 8–10, 39, 62, 90–93, 112, 124 |
| | | THOV | | | 7, 125 |
| | | DHOV | | | 126 |
| | | BKNV | | | 127 |
| Mouse Mx2 | Cytoplasm | VSV | Rhabdoviridae | ssRNA (−) | 11, 12 |
| | | HTNV | Bunyaviridae | ssRNA (−) | 13 |
| Human MxA | Cytoplasm | Influenza virus | Orthomyxoviridae | ssRNA (−) | 5, 10, 14, 91 |
| | | THOV | | | 14, 18, 22, 24, 105 |
| | | VSV | Rhabdoviridae | ssRNA (−) | 14–16, 21, 68, 111, 113 |
| | | Rabies virus | | | 128 |
| | | HTNV | Bunyaviridae | ssRNA (−) | 19 |
| | | LACV | | | 19, 23, 24, 27 |
| | | CCHFV | | | 26 |
| | | RVFV | | | 19, 129 |
| | | PUUV | | | 20 |
| | | TULV | | | 20 |
| | | Measles virus | Paramyxoviridae | ssRNA (−) | 17, 115 |
| | | SFV | Togaviridae | ssRNA (+) | 21, 24 |
| | | CSFV | Flaviviridae | ssRNA (+) | 28 |
| | | IBDV | Birnaviridae | dsRNA | 30 |
| | | Reovirus | Reoviridae | dsRNA | 30 |
| | | HBV | Hepadnaviridae | dsDNA | 25, 120 |
| | | ASFV | Asfarviridae | dsDNA | 29 |
| Human MxB | Nucleus | None | | | 15, 18 |
| Rat Mx1 | Nucleus | Influenza virus | Orthomyxoviridae | ssRNA (−) | 71, 130 |
| | | THOV | | | 130 |
| | | VSV | Rhabdoviridae | ssRNA (−) | 71 |
| Rat Mx2 | Cytoplasm | VSV | Rhabdoviridae | ssRNA (−) | 71, 130, 135 |
| | | LACV | Bunyaviridae | ssRNA (−) | 131 |
| | | RVFV | | | 130 |
| Rat Mx3 | Cytoplasm | None | | | 71, 130, 131 |
| Chicken Mx | Cytoplasm | None | | | 132, 133 |
| | | Influenza virus | Orthomyxoviridae | ssRNA (−) | 134 |
| | | VSV | Rhabdoviridae | ssRNA (−) | 134–136 |
| | | NDV | Paramyxoviridae | ssRNA (−) | 136 |
| Duck Mx | Nucleus and cytoplasm | None | | | 87 |
| Cow Mx1 | Cytoplasm | VSV | Rhabdoviridae | ssRNA (−) | 137, 138 |
| | | Rabies virus | | | 128 |
| Cow Mx2 | Cytoplasm | VSV | Rhabdoviridae | ssRNA (−) | 118, 138 |
| Pig Mx1 | Cytoplasm | Influenza virus | Orthomyxoviridae | ssRNA (−) | 107 |
| | | VSV | Rhabdoviridae | ssRNA (−) | 139 |
| Dog Mx1, Mx2 | Cytoplasm | VSV | Rhabdoviridae | ssRNA (−) | 140 |
| Atlantic salmon Mx1-Mx3 | Cytoplasm | ISAV | Orthomyxoviridae | ssRNA (−) | 33, 38 |
| | | IPNV | Birnaviridae | dsRNA | 34 |
| Japanese flounder Mx | Cytoplasm | HIRRV | Rhabdoviridae | ssRNA (−) | 31 |
| | | VHSV | | | 31 |
| Grouper Mx1-Mx3 | Cytoplasm | YGNNV | Nodaviridae | ssRNA (+) | 32, 35 |
| Senegalese sole Mx | | Aquabirnavirus | Birnaviridae | dsRNA | 141 |
| Barramundi Mx | Cytoplasm | NNV | Nodaviridae | ssRNA (+) | 37 |
| | | IPNV-SP | Birnaviridae | dsRNA | 147 |
| Seabream Mx1-Mx3 | | IPNV | Birnaviridae | dsRNA | 119 |
| Rare minnow Mx | | GCRV | Reoviridae | dsRNA | 36 |
| Rainbow trout Mx1 | Cytoplasm | IPNV | Birnaviridae | dsRNA | 142 |
| | | SAV | Togaviridae | ssRNA (+) | 142 |

virus infection (3, 39). This resistance is inherited as a dominant autosomal trait and is dependent on a single gene (*Mx1*) located on chromosome 16 (40). Most inbred mouse strains carry an inactive *Mx1* gene due to deletion of three exons or the presence of a nonsense mutation leading to premature termination of translation (41). IFN induces the expression of *Mx1* and is required for

the resistance of A2G mice to influenza A virus: treatment of these mice with an interferon-neutralizing antiserum renders them susceptible to the virus (42). The IFN inducibility of the *Mx1* gene facilitated the isolation of the Mx1 protein by comparison of the proteins derived from *in vitro*-translated mRNAs isolated from mouse strains that are sensitive or resistant to influenza virus in-

fection (43). However, Mx1 can also protect cells against influenza virus in the absence of other IFN-induced proteins (4, 24, 44).

*Mx1* orthologs (related genes in other species that evolved from a common ancestral gene) and paralogs (related genes that originated by duplication within a genome) were later identified in many different animal species. The first homologous Mx protein was observed in human peripheral blood lymphocytes treated with type I IFN. This protein, called MxA and encoded by *MX1*, was isolated by immunoprecipitation with a monoclonal antibody (2C12) raised against the mouse Mx1 protein (45). By studying the structures of these *Mx* genes, it became clear that mice and humans carry more than one *Mx* gene (46). Remarkably, the second mouse *Mx* gene is also not functional in most inbred mouse strains, in contrast to the intact *Mx2* gene in wild mouse species (11). Subsequent analysis of human cDNA clones derived from type I IFN-treated human fibroblasts led to the discovery of MxB, encoded by *MX2*, the second human *Mx* gene (47).

The strategies used to identify homologous Mx proteins in mouse and human cells were based on the assumption that the corresponding *Mx* genes are induced by IFN. Subsequent detailed study of IFN inducibility showed that the *Mx* genes are induced by type I (IFN-α and IFN-β) or type III (IFN-λ) IFNs (45, 47–50); the *Mx* promoter does not respond to IFN-γ (type II IFN), interleukin-1α (IL-1α), or tumor necrosis factor alpha (TNF-α). Upregulation of *Mx* expression in response to virus infection is indirect and depends on the production of type I or type III IFNs (47, 49–51). After the discovery of mouse and human Mx proteins, it became clear that Mx proteins are present in almost all vertebrates and that all of them are induced by IFN. For example, the chicken *Mx* gene can be induced by IFN, poly(I) · (C) and UV-treated Newcastle disease virus (NDV) (52). In pregnant cattle, *Mx* gene expression can be induced not only by the antiviral type I IFN but also by IFN-τ (a type I IFN produced by cattle during pregnancy) (53, 54). Likewise, fish Mx proteins are expressed in response to type I IFNs (e.g., induction by poly(I) · (C) or by virus infection) but not to type II IFNs or lipopolysaccharide (LPS) (55, 56). In summary, *Mx* genes are conserved in vertebrates and are typically induced by type I and III IFNs, and Mx proteins are effectors of the antiviral state.

## STRUCTURE OF Mx PROTEINS

Mx proteins are members of the family of large GTPases, which includes dynamins (57, 58). These GTPases share an N-terminal GTPase domain, a middle domain (MD), and a C-terminal GTPase effector domain (GED) (Fig. 1A) (57, 58). Unlike classical dynamins, Mx proteins lack a pleckstrin homology domain (involved in membrane targeting) and a proline-rich domain (involved in protein-protein interactions) (58).

### GTPase Domain

The GTPase domain is the most conserved part of Mx proteins and other members of the dynamin family of large GTPases. In Mx proteins, this domain consists of a tripartite GTP-binding motif (GDXXSGKS, DLPG, and TKPD) and a dynamin signature (LP RXXGXXTR) (Fig. 1A) (59–61). The dynamin signature contains residues that coordinate the $Mg^{2+}$ ion required for GTPase activity (61). The first two GTP-binding motifs, which flank the dynamin signature, bind the phosphate moiety of GTP, and the third GTP-binding motif is important for binding guanosine (62, 63). The second and third GTP-binding motifs

are perfectly conserved in different species, whereas the first GTP-binding domain shows some differences between species (Fig. 1A). The dynamin signature is not completely conserved, but nearly all known Mx proteins (turkey Mx is an exception) contain the penultimate threonine residue, which is important for coordination of the $Mg^{2+}$ ion. This strong conservation is probably because the biological activity of Mx proteins requires an intact GTPase domain (63, 64).

### Middle Domain and GTPase Effector Domain

In the primary structure, the GTPase domain is followed by the middle domain (MD) (also called central interactive domain [CID]) and the C-terminal GTPase effector domain (GED) (65–67). Both the MD and GED are important for the conformation and activity of Mx proteins (65–67). The MD is important for oligomerization and virus target recognition (65, 68). On the other hand, the GED functions as an intramolecular GTP-activating domain: the C-terminal leucine zipper motif (65 to 70 amino acids) in the GED folds back to join the N-terminal GTP-binding domain, forming the enzymatically active center of Mx proteins. Mutants lacking the GED domain have no detectable GTPase activity (69). The C-terminal leucine zipper is evolutionarily conserved, suggesting a vital role in Mx function (Fig. 1A). The C terminus of rodent Mx1 protein is unique in that it contains a functional nuclear localization signal (NLS) (amino acids 606 to 615) (70, 71). Mouse Mx1 accumulates in the nuclei of most cell types tested, and this localization is important for its antiviral activity (70, 72).

### Three-Dimensional Structure of Mx Proteins

The crystal structure of GTP-free human MxA protein was recently resolved (65, 73) and shown to resemble the three-dimensional (3D) structure of dynamin proteins (74). It is remarkable that crystallized MxA adopts a three-domain structure that does not coincide with the domains identified in the primary amino acid sequence. In the crystal structure, the GTPase domain is connected to the bundle signaling element (BSE) by a hinge, and the BSE is connected to the stalk by a second hinge (Fig. 1B) (73). The GTPase domain comprises a central β-sheet surrounded by α-helices. The BSE consists of three α-helices, each of which is derived from a different part of the MxA primary structure. One α-helix contains the amino acids just before the GTPase domain, the second one separates the GTPase domain from the MD, and the third corresponds to the C-terminal part of the GED (Fig. 1A) (73). Therefore, formation of the BSE requires backfolding of the C-terminal leucine zipper to the N-terminal GTPase domain. Concomitantly, three α-helices from the MD and one from the GED (preceding the carboxy-terminal BSE α-helix) adopt a helix-rich conformation known as the stalk (65, 73). Thus, the C-terminal leucine zipper is part of both the stalk and the BSE domains. This interaction between the C-terminal leucine zipper and the MD was originally proposed by Di Paolo et al. on the basis of two-hybrid experiments using deletion mutants of human MxA (75). The human MxA is the only Mx protein crystallized so far, but its strong structural similarity to dynamin, another large GTPase family member, and the strong conservation between Mx proteins suggest that other Mx proteins likely adopt a similar three-dimensional structure (73, 74).
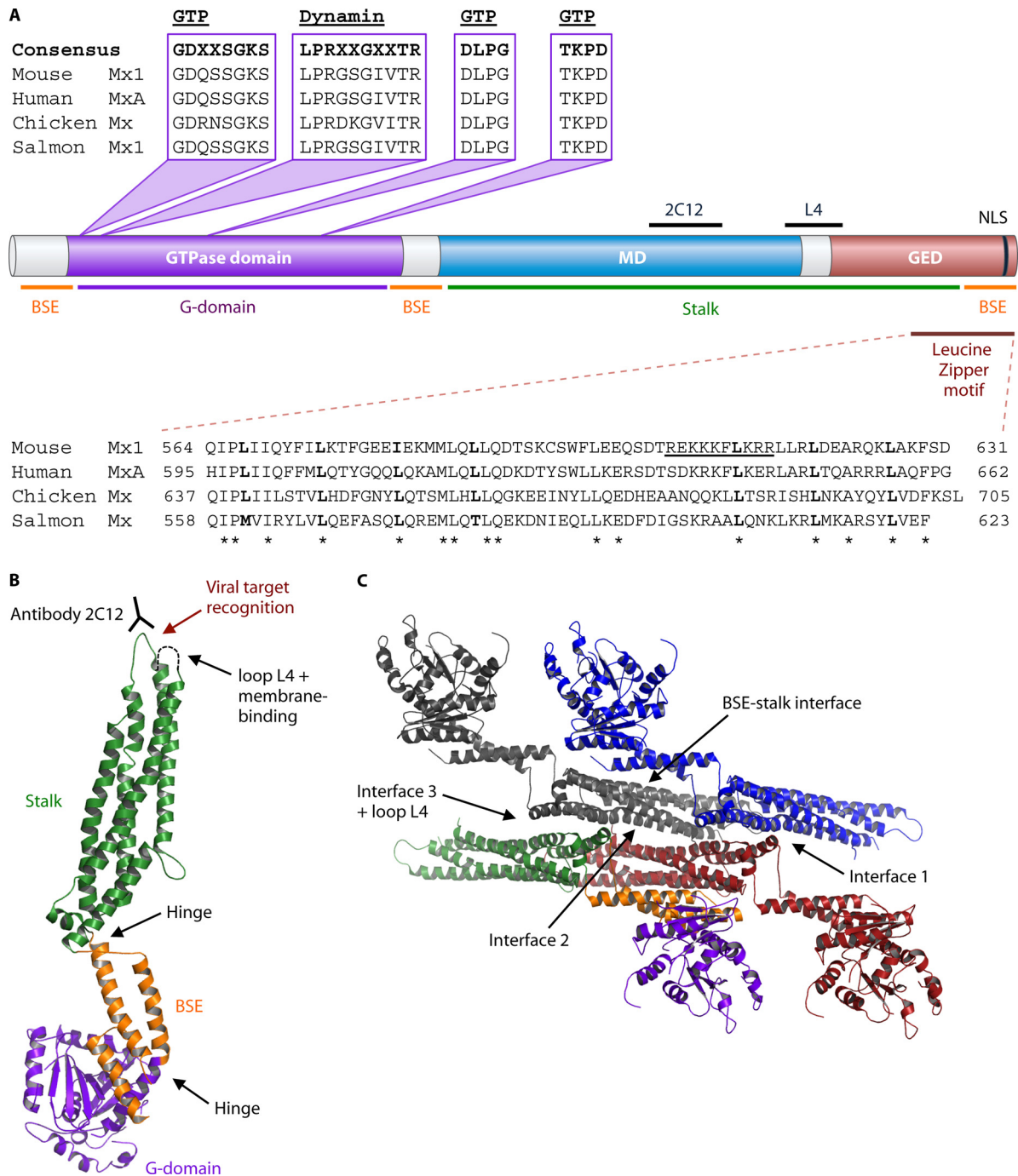
**FIG 1** Structure of Mx proteins. (A) The different domains of Mx proteins delineated by their primary sequence and their relationship to the domains determined in the 3D structure of Mx proteins. The Mx domains in the primary sequence include the GTPase domain, the middle domain (MD), and the GTPase effector domain (GED). The domains in the 3D structure include the G domain, the bundle signaling element (BSE), and the stalk domain. The amino acid sequences of domains important for the antiviral activity of Mx proteins are aligned. These domains include the GTP-binding motif, the dynamin signature, the C-terminal leucine zipper, and the nuclear localization signal (NLS). The conserved amino acids in the leucine zipper are indicated with an asterisk. The position of the epitope recognized by the monoclonal antibody 2C12, which can neutralize Mx antiviral activity, is indicated above the MD. The 2C12 epitope and loop L4 are important for viral target recognition. (B) 3D structure of Mx proteins (PDB file MxA 3SZR [73]). (C) 3D structure of MxA tetramer showing the interfaces (1 to 3 and loop L4) involved in oligomer formation (65, 73, 109). The images in panels B and C were generated with PyMOL.

## Oligomerization of Mx Proteins

At low protein concentrations and physiological salt concentrations, Mx proteins form tetramers in solution. At higher protein concentrations (>1.5 mg/ml), these tetramers oligomerize fur-

ther into large filaments and rings. These structures have been observed by electron microscopy and have been characterized by size exclusion chromatography and sedimentation assays (66, 67, 76–78). Mx oligomerization is mediated by three interfaces and

one loop region (L4) (Fig. 1C). These interfaces mediate a criss-cross interaction pattern between the stalk domains of different Mx molecules (65, 75), which ultimately results in ring formation. In these Mx rings, the stalk domains point inwards and the GTPase domains are located at the periphery. In addition to the stalk-stalk interactions, MxA oligomerization is also mediated by interactions between the BSE and the stalk of a neighboring parallel MxA monomer. These BSE-stalk interactions (Fig. 1C) are crucial for transmitting conformational changes, caused by GTPase activity, from the GTPase domain to the stalk domain of a neighboring MxA molecule. This cross talk is required for antiviral activity of Mx proteins (65, 73). Furthermore, when two Mx rings interact laterally (like two millstones), the GTPase domains of Mx molecules in adjacent rings interact with each other in the presence of GTP or its analogs (GTPγS or GDP/AlF$_4^-$) (67). This second-order oligomerization step stimulates GTPase activity by a cooperative mechanism.

The antiviral activity of MxA depends on both oligomerization and GTPase activity. However, mutations that impair oligomerization by inhibiting stalk-stalk or BSE-stalk interactions enhance the GTPase activity of MxA, probably by relieving structural constraints of Mx oligomerization on GTPase activity (65, 73). This suggests that antiviral activity requires an optimal balance between a rigid conformation (for interaction with the viral target) and mobility (allowing a stronger GTPase activity due to interactions between GTPase domains). Thus, although GTPase activity is required, stronger GTPase activity does not necessarily correlate with stronger antiviral activity (73).

### Membrane Binding of Mx Proteins

Though MxA has no pleckstrin homology domain, it can associate with cellular membranes by binding to negatively charged lipids. This binding is mediated by a loop consisting of four lysine residues (MxA residues 554 to 557) located at the tip of the stalk region, distal from the G domain (Fig. 1B). MxA preferentially associates with negatively charged phospholipids, probably because of electrostatic interaction with the positively charged lysine residues in the membrane-binding loop (79). Unfortunately, this loop is unstructured and not visible in the crystal structure (65, 73). The affinity for membrane lipids supports self-assembly of dynamin and MxA, but this lipid binding leads to enhanced GTPase activity only in the case of dynamin (76, 79, 80).

In the cell, MxA associates with the smooth endoplasmic reticulum (ER) and the smooth ER-Golgi intermediate (27, 76, 80), which may contribute to antiviral activity. Membrane binding might facilitate contact with viral target structures, for example, with the nucleocapsid protein of La Crosse virus (LACV). Both MxA and the nucleocapsid protein of LACV localize to the smooth ER, which could facilitate their initial contact and interaction. This interaction leads to sequestration of the viral nucleocapsid protein to membrane-associated, large perinuclear complexes (27). Membrane binding might also lead to formation of a depot of MxA molecules that are protected from proteolytic attack: the membrane-binding loop contains a proteinase K cleavage site, which is protected from cleavage after membrane binding (79). The membrane-binding loop is fairly conserved in mammalian Mx1 proteins, which may indicate that membrane binding plays a role in antiviral activity.

## PHYLOGENETIC ANALYSIS OF Mx FAMILY MEMBERS

Mx genes are present in nearly all sequenced chordate genomes; a notable exception is the opossum (Monodelphis domestica) genome. We used the presence of the conserved N-terminal GTPase domain, an MD, and a C-terminal GED (Fig. 1A) in the Mx gene products to identify and compare Mx family members across a large set of vertebrate species (58). We identified Mx genes in mammals, monotremes, birds, amphibians, reptiles, and fish and determined their phylogenetic relationships. The Mx protein sequences (n = 60) were aligned in ClustalX2 (81) to estimate their phylogenetic relationships. The identified Mx protein sequences range from 620 amino acid residues (Japanese flounder [Paralichthys olivaceus]) to 721 amino acid residues (duck [Anas platyrgynchos]). Because the N-terminal region preceding the GTPase domain is highly variable, we excluded it from the alignment used to build the phylogenetic tree. The deduced phylogenetic tree reveals different subfamilies of Mx proteins that segregate into three classes: (i) fish; (ii) amphibians, reptiles, and birds; and (iii) mammals (Fig. 2).

The evolutionarily most ancient Mx genes are from lamprey and amphioxus (Fig. 2, top). Depending on the species, fish have one to nine Mx genes. The multiple Mx genes in fish probably arose by gene duplications, as the encoded proteins are more closely related to their paralogs than to their orthologs in other fish species. Birds, reptiles, and amphibians have single Mx genes that presumably resemble the ancestor of mammalian Mx genes, which duplicated into two paralogous Mx genes soon after the emergence of mammals. As a result, most mammals have two Mx genes: MX1 and MX2. In humans and other primates, the gene products derived from MX1 and MX2 are usually named MxA (= Mx1) and MxB (= Mx2). Remarkably, in the phylogenetic tree the two rodent Mx proteins segregate together as a branch that is most closely linked to Mx1 proteins of other mammals (Fig. 2).

We also identified the genes flanking the Mx genes in the different species to look for conserved synteny (colocalization and sequential arrangement of related genes on a chromosome of a different species). We collected data from the UCSC, NCBI Map Viewer, and Ensembl genome browsers but excluded fish genomes because only a few fish genome sequences are available. In addition, fish Mx loci are much more complex and we found no evidence for conserved synteny to Mx genes from other vertebrates. The Mx genes from amphibians to mammals are all flanked by the FAM3B and TMPRSS2 genes, although the positions and orientations of the Mx genes relative to those of FAM3B and TMPRSS2 are occasionally shuffled (Fig. 3). The TMPRSS2 gene encodes a serine protease that can process the influenza A virus hemagglutinin into its fusion-competent state in human airway epithelial cells (82), and the FAM3B gene encodes the pancreatic secreted cytokine FAM3B (83). There is no obvious functional relationship to explain this chromosomal synteny of the Mx locus across different species ranging from amphibians to human.

The Mx locus in rodents is rearranged in comparison to the Mx loci of other mammals (Fig. 3). During evolution, rodents presumably lost their ancestral Mx2 gene. Based on sequence homology, the gene that is now named Mx2 in mouse was likely derived from an ancestral Mx1-like gene, and the mouse Mx1 evolved from that ancestral gene after duplication. This hypothesis is supported by the fact that the two rodent Mx proteins are more related to Mx1 than to Mx2 proteins from other mammals (Table 2).
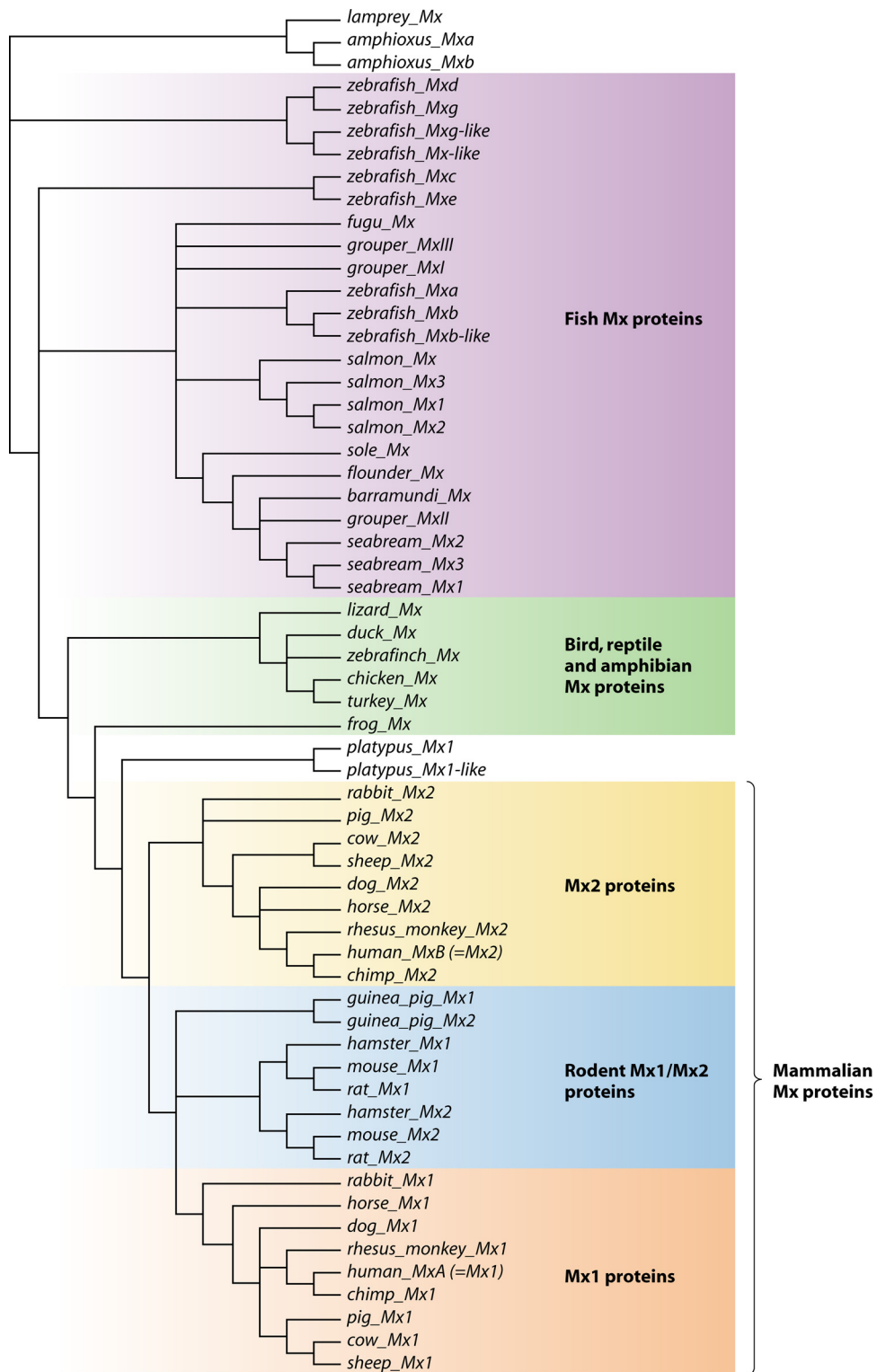
**FIG 2** Phylogenetic tree based on primary amino acid sequences of Mx proteins. The Mx protein sequences were aligned in ClustalX2 (using the protein weight matrix PAM350), and the nonconserved N terminus was trimmed using the program Jalview (81, 143). This alignment was then used to build a bootstrap neighbor-joining tree in ClustalX2 with 1,000 bootstrap replicates. The deduced phylogenetic tree, visualized with Dendroscope (144), allows the identification of different subfamilies of Mx proteins: (i) fish Mx proteins (purple); (ii) Mx proteins of birds, reptiles, and amphibians (green); (iii) nonrodent mammalian Mx2 proteins (yellow); (iv) rodent Mx1/Mx2 proteins (blue); and (v) nonrodent mammalian Mx1 proteins (orange).
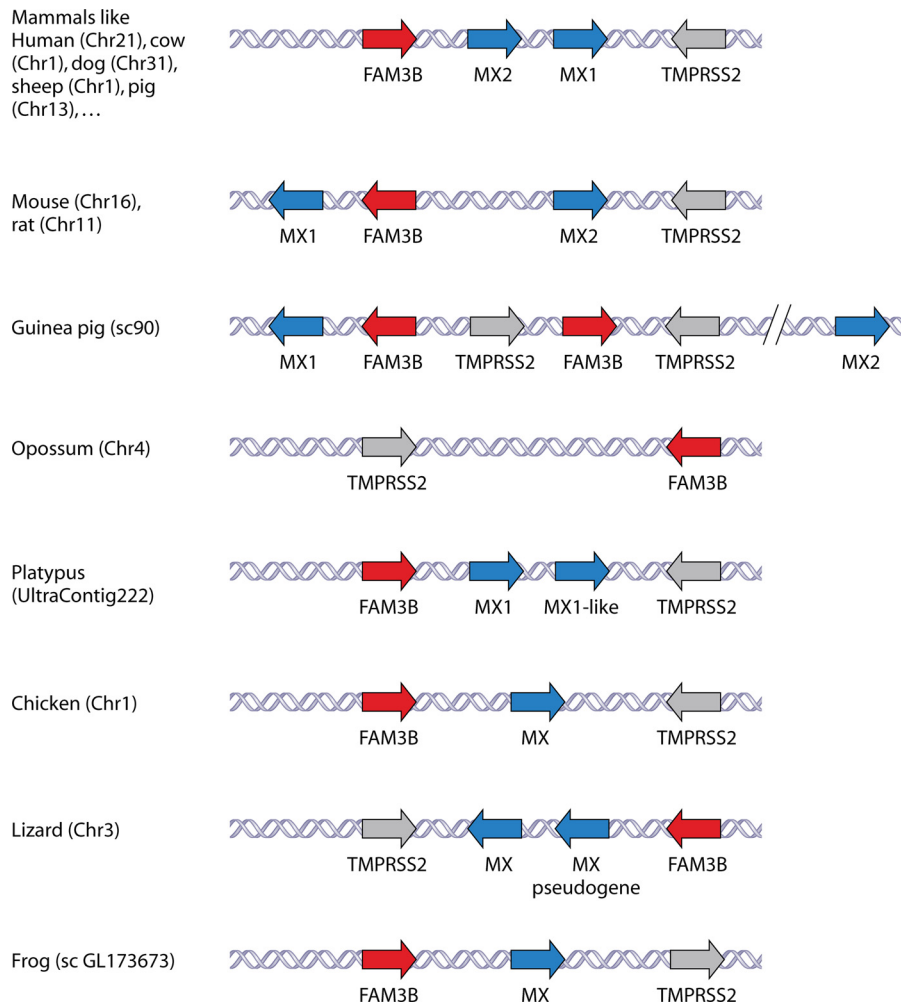
**FIG 3** Genomic organization of *Mx* genes. The genomic organization of the different *Mx* genes of the different organisms was retrieved from the UCSC, NCBI Map Viewer, and Ensembl genome browsers; their organization with respect to their flanking genes *FAM3B* and *TMPRSS2* is shown. The chromosome (Chr), scaffold (sc), or contig number is given.

The mouse and rat *Mx1* and *FAM3B* genes are oriented in opposite directions relative to the *Mx2* gene, suggesting that a duplication of the ancestral *Mx2* gene was accompanied by rotation of the *FAM3B* gene during the evolution of rodents. In rats, three Mx proteins have been reported (84). They are named Mx1, -2, and -3, but the genes encoding Mx2 and Mx3 are most likely allelic variants of the same gene, as only two *Mx* genes are found in the rat genome. Compared to those of mouse and rat, the guinea pig *Mx* locus seems to lack the orthologous *Mx2* gene, and the *FAM3B* and *TMPRSS2* genes are duplicated. However, the guinea pig *Mx2* gene is located approximately 4.3 Mb further away on the same

chromosome. The chromosomal arrangement of the two platypus *Mx* genes resembles that in other nonrodent mammals. However, the predicted gene products are more closely related to each other than to other mammalian Mx proteins. Birds, reptiles, and amphibians have one *Mx* gene, flanked by *FAM3B* and *TMPRSS2* genes (Fig. 3). The lizard *Anolis carolinensis* has an additional *Mx*-like gene, but there is no evidence that this gene is transcribed, and presumably it is a pseudogene.

The genetic relationship between the Mx proteins of different organisms can provide clues about the antiviral spectrum and the presence of specific functions in different subclasses of Mx proteins. For example, the Mx1 proteins from rodents are the only ones known to have an NLS close to the C terminus. This NLS is important for their antiviral activity against different viruses, including orthomyxoviruses (72). Another example is the variable and sometimes extended N-terminal domain that precedes the GTPase domain in Mx2 proteins in mammals (except rodents) and Mx proteins in birds. This N-terminal part of human MxB contains a functional NLS and a proline-rich region, which are absent in human MxA. Human MxB localizes in the heterochro-

**TABLE 2** Sequence similarity between human MxA/MxB and mouse Mx1/Mx2

| Protein | % Sequence similarity with: | | |
|---|---|---|---|
| | Human MxB | Mouse Mx1 | Mouse Mx2 |
| Human MxA | 77.7 | 81.9 | 88.1 |
| Human MxB | | 73.3 | 76.3 |
| Mouse Mx1 | | | 83.5 |

matin region beneath the nuclear envelope, unlike its paralog MxA, which is cytoplasmic (85). This extended N terminus was probably lost during the evolution of mammalian Mx1 proteins. Though the expression of human MxB depends on type I IFN, no antiviral activity has yet been described for human MxB. Instead, this protein is involved in cellular processes, such as nuclear import and cell cycle progression (86). Related Mx2 proteins without known antiviral activity might be involved in similar cellular processes in other mammals. Duck Mx localizes in both the cytoplasm and the nucleus, but no nuclear localization motifs are apparent in the primary sequence. The N terminus of duck Mx might also harbor an NLS, but the importance of the N terminus for nuclear localization needs to be determined (87).

## ANTIVIRAL MECHANISMS OF Mx PROTEINS

The antiviral activities of Mx proteins of different species have been studied for many RNA viruses (Table 1). Figure 4 depicts examples of different steps in the life cycles of a selection of viruses that are repressed by Mx proteins. How Mx protein family members are thought to hinder viral replication is amazingly diverse. We provide an overview of these proposed mechanisms of action, according to the virus family that is targeted.

### Antiviral Activity against *Orthomyxoviridae*

**Influenza virus.** The reference virus for the antiviral activity of the Mx proteins is influenza virus, and different Mx proteins can inhibit influenza virus replication. The mouse *Mx1* was the first (ortho)myxovirus resistance gene to be discovered (3, 39). The effect of mouse Mx1 expression on different steps of influenza virus replication has been studied extensively, yet the exact antiviral mechanism remains unclear. Mouse Mx1 does not seem to affect the uncoating of the virus or the transport of viral ribonucleoproteins (vRNPs) into the nucleus (88–90). Infection of IFN-stimulated cells, and therefore Mx1-expressing cells, resulted in reduced primary viral transcription and viral mRNA translation (2, 90). A caveat of these two studies is that the antiviral activity of the mouse Mx1 protein was combined with other IFN-dependent antiviral effects. Although both studies showed that IFN treatment inhibits transcription and translation, the part attributed to the Mx1 protein was assigned differently: in IFN-treated macrophages the presence of Mx1 affected mainly viral translation, whereas in IFN-treated embryonic fibroblasts it affected mostly primary transcription (2, 90). Pavlovic and colleagues confirmed that mouse Mx1 suppresses primary transcription of influenza A virus genes by using a stable fibroblast cell line that constitutively expresses mouse Mx1, thereby excluding IFN-dependent effects on viral replication (91). Mx1 differentially inhibits the transcription of the different influenza virus segments. It strongly affects the largest transcripts (encoding PB1, PB2, and PA) and moderately affects the hemagglutinin (HA) and NP transcripts, but it hardly affects the transcripts generated from the M and NS segments. This correlation between the degree of inhibition and the length of the segment suggests that Mx1 hampers transcriptional elongation rather than initiation (2, 91). The inhibitory effect of mouse Mx1 has often been studied by using a minireplicon system with a reporter gene as a readout (5, 10, 92). This assay indicates that mouse Mx1 targets the influenza vRNPs. Two subunits of the ribonucleoprotein complex have been suggested as targets for mouse Mx1 activity. The first is the PB2 protein, as its overexpression can outcompete the activity of Mx1 (92,

93). The second is the NP protein, because the sensitivities of different influenza A virus strains to mouse Mx1 and human MxA depend on their NP proteins: virus strains of human origin are more resistant to the effects of Mx1 and MxA than strains of avian origin (5, 10, 94). Recently, Mänz and colleagues (95) identified the parts in NP that are responsible for Mx sensitivity in the 1918 and 2009 pandemic virus strains (A/Brevig Mission/1/1918 and A/Hamburg/4/2009, respectively). In both cases, a minimal set of three residues was important for increased Mx1 (and MxA) resistance (positions 100/283/313 and 53/100/313 for the 1918 and 2009 pandemic viruses, respectively). These residues are grouped in separate but overlapping regions in the body domain of the NP protein (Fig. 5). These residues are solvent exposed and therefore readily accessible for cellular factors, such as the Mx1 protein (95). We recently demonstrated that Mx1 interacts with both the NP and PB2 proteins and disturbs their interaction by a mechanism that depends on GTP binding and/or GTPase activity. Surprisingly, the interactions of Mx1 with NP and PB2 do not depend on GTPase activity, which is essential for its antiviral activity (94). In the future, it will be interesting to determine if a higher Mx resistance of specific NP variants correlates with a weaker binding to Mx1 (and MxA) to confirm the crucial role of influenza virus NP in Mx1 sensitivity. Taken together, these results suggest that Mx1 targets vRNPs, possibly at the NP-polymerase interface, leading to disruption of the vRNPs and inhibition of influenza virus infection. It will be interesting to confirm these interactions in a cell-free system using purified, enzymatically active vRNPs and Mx1 and to demonstrate that in this system Mx1 can disrupt the PB2-NP interaction.

Human MxA localizes to the cytoplasm, and, like Mx1, it has been extensively studied as a suppressor of influenza virus infection. For MxA, this suppression occurs at an ill-defined step after viral primary transcription but before viral protein expression (15, 91). When the MxA protein is directed to the nucleus by making a fusion with a heterologous NLS (e.g., that of the simian virus 40 [SV40] large T antigen), its mode of action becomes similar to that of the mouse Mx1 protein in that it also blocks primary transcription of influenza virus genes (96). In contrast to the human MxA protein, which can inhibit influenza virus replication inside the cytoplasm and the nucleus, the mouse Mx1 protein is active only inside the nucleus. This restriction for mouse Mx1 was demonstrated by using a cytoplasmic mutant of Mx1 (R614E, mutated to resemble MxA) that has no anti-influenza virus (or anti-VSV) activity. When this protein was translocated back to the nucleus by a heterologous NLS, it regained its antiviral activity (72).

Why does mouse Mx1 have to be in the nucleus to exert its antiviral activity? It is possible that mouse Mx1 needs another cellular factor that is present only in the nucleus or that it is post-translationally modified by a nuclear protein. For example, the RNA helicase UAP56 (or URH49) might act as a bridging protein, as it interacts with NP and with mouse Mx1 and human MxA (97, 98). However, this helicase is not restricted to the nucleus and partially redistributes to the cytoplasm in the presence of human MxA (98), which argues against the hypothesis that it could be responsible for the strict nuclear activity of the mouse Mx1 protein. Other potential bridging factors that bind to influenza virus vRNP components include nucleophosmin (99), CDK9 (100), USP11 (101), and Hsp90 (102, 103). It is also possible that mouse Mx1 recognizes viral targets that adopt the right conformation only in the nucleus. These viral targets are probably the incoming
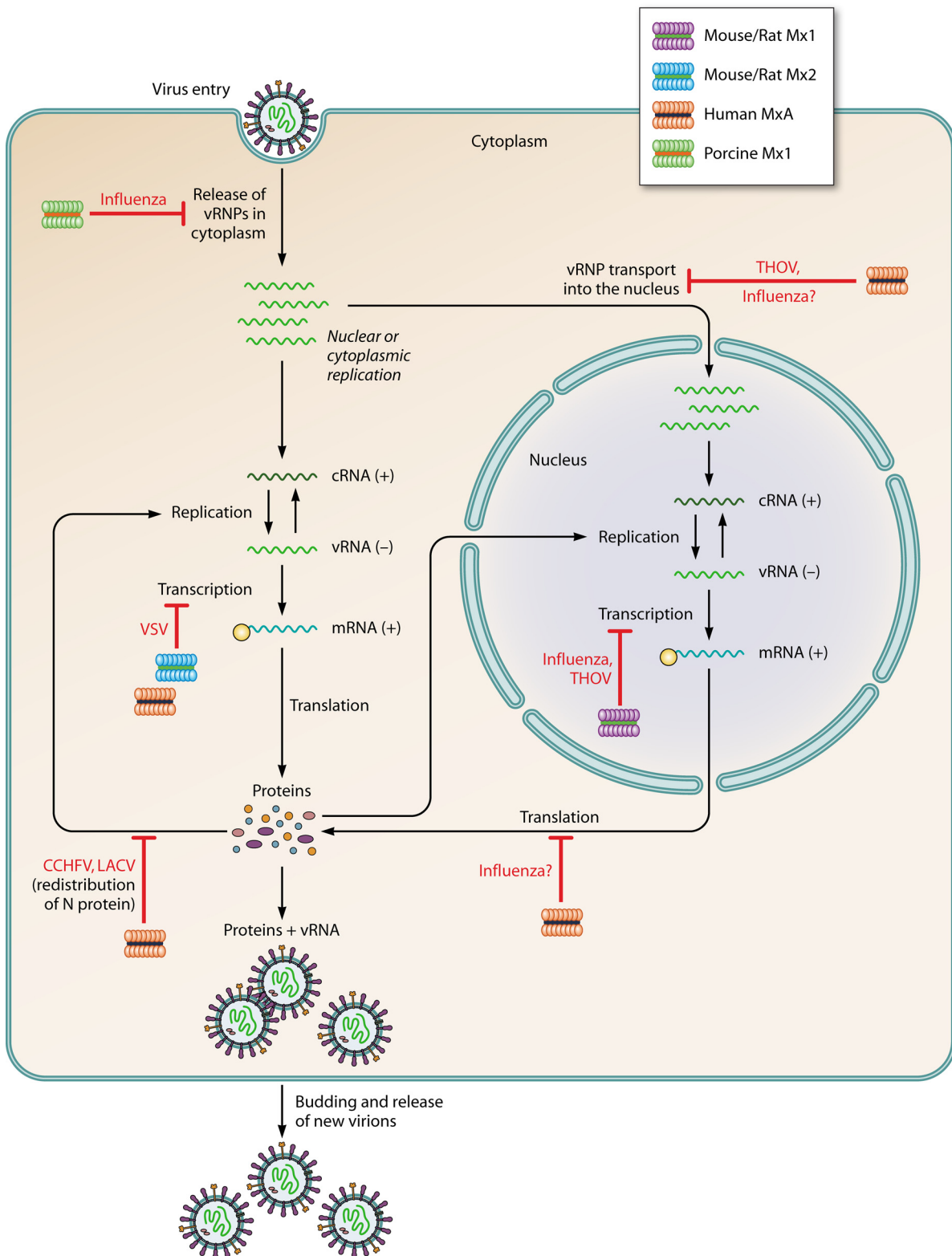
**FIG 4** Antiviral mechanisms of Mx proteins. Steps in the life cycles of different viruses that are inhibited by Mx proteins are shown. Human MxA, mouse Mx2, and porcine Mx1 inhibit Thogoto virus (THOV), vesicular stomatitis virus (VSV), La Crosse virus (LACV), and influenza viruses in the cytoplasm. Mouse Mx1 inhibits THOV and influenza viruses in the nucleus.
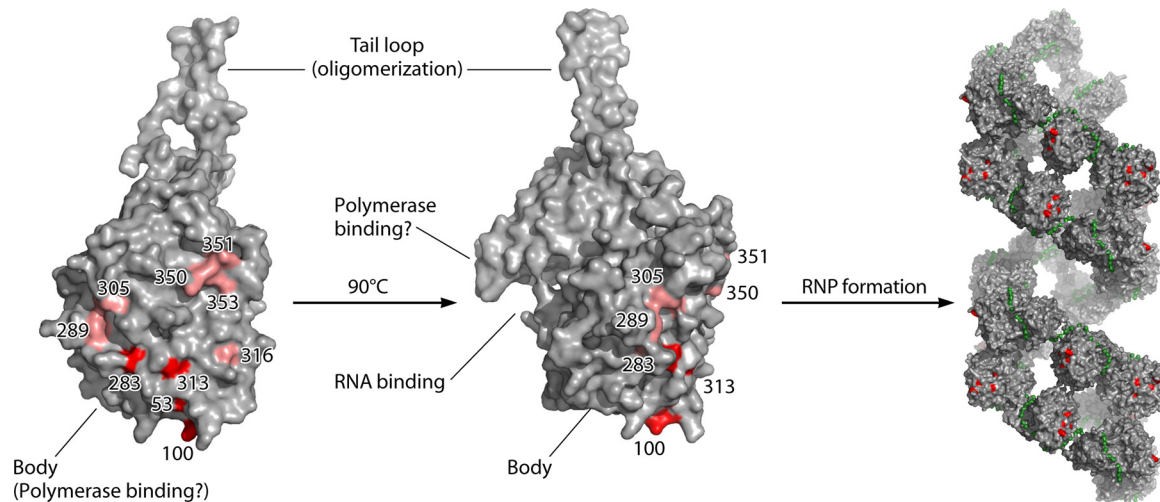
**FIG 5** Amino acid residues in influenza A virus nucleoprotein associated with sensitivity to Mx. Left, 3D structure of an NP monomer (PDB file 2Q06 [145]). The residues important for Mx1 and MxA resistance are highlighted in red. Residues that contribute in only a minor way to Mx1 resistance are indicated in light red (95). Right, these important residues are also exposed on the surface of viral ribonucleoprotein (RNP) complexes (PDB file 4BBL [146]). The RNA strand is depicted in green. The images were generated with PyMOL.

vRNPs of orthomyxoviruses, which are transported to the nucleus following their release in the cytoplasm after viral entry (2, 91). Apparently, these vRNPs cannot be recognized by the cytoplasmic mouse Mx1 R614E mutant when they are transported to the nuclear pore. Recognition of these vRNPs by Mx1 might require conformational or structural changes during or after their transport to the nucleus. Although experimental evidence is missing, the recently resolved crystal structure of MxA led to the suggestion that human MxA blocks influenza virus by recognizing the incoming vRNPs, followed by self-assembly of MxA rings around these vRNPs (73, 104). This mechanism is comparable to that for the recognition and inhibition of THOV (22, 105).

Nuclear transport of orthomyxovirus vRNPs is dependent on an NLS on their main structural component, the NP protein. The NP protein is not the only viral protein that interacts with the nuclear import machinery. The interaction between the viral PB2 and NP proteins and different importin alpha isoforms is also important for virus replication (106). If, as proposed, NP and PB2 are targets for the Mx1 protein, their colocalization at nuclear pores could be important for the antiviral activity of the Mx1 protein. Similarly, MxA could recognize both proteins at the cytoplasmic face of the nuclear pores. In this way, Mx proteins could function as antiviral gatekeepers at the nuclear membrane, blocking vRNPs just before or after nuclear entry.

The similarity in anti-influenza virus mechanisms of mouse Mx1 and MxA targeted to the nucleus, together with the similar Mx resistance phenotypes of certain virus strains, indicates that both Mx proteins recognize the same viral target, most likely the NP protein in vRNP structures. This is in line with the demonstrated interaction between (nuclear) MxA and influenza virus NP after cross-linking (96).

In contrast to mouse Mx1, which does not recognize the influenza virus vRNPs inside the cytoplasm, the pig Mx1 protein recognizes these vRNPs and somehow prevents their transport into the nucleus (107). However, pig Mx1 works indirectly by influencing the endocytic pathway, leading to a delayed and centripetal movement of viral particles to the nucleus (107).

**THOV.** Another member of the *Orthomyxoviridae* that is inhibited by Mx proteins is Thogoto virus (THOV), a natural pathogen of mice (7, 14, 18, 22, 105, 108). THOV is very sensitive to human MxA activity, and the antiviral mechanism was resolved by Kochs and colleagues (22, 105). Human MxA blocks a very early step in the viral life cycle by preventing the transport of THOV vRNPs into the nucleus. This blockage is mediated by the interaction of MxA with RNA-bound NP; this interaction probably shields the signals responsible for nuclear translocation of the vRNPs (22, 105). The NP interaction interface in MxA was mapped to its C terminus, in a domain that includes loop L4 (105, 109). The interaction between MxA and THOV NP was also blocked by the 2C12 antibody, which recognizes an epitope in the C terminus, confirming the importance of the C terminus for NP binding (22, 68, 105). MxA had no effect on other steps in the viral life cycle, such as protein synthesis or transport of newly produced proteins into the nucleus. In contrast, the nuclear forms of human MxA and probably also mouse Mx1 block THOV polymerase activity in the nucleus without affecting vRNP transport (108). This demonstrates that the antiviral mechanism of Mx proteins is determined at least in part by their subcellular localization. It is possible that the mouse Mx1 protein inhibits the THOV polymerase complex like it inhibits the influenza virus polymerase complex, but this has not been demonstrated.

**Common features of Mx antiviral activity against *Orthomyxoviridae*.** Inhibition of orthomyxoviruses by Mx proteins generally requires an intact GTPase domain. This has been demonstrated by examining the antiviral activities of different GTPase-inactive mutants (63, 64, 110). For example, the K49A mutation in the first GTP-binding motif of mouse Mx1 prevents GTP binding and subsequent GTPase activity. This mutant Mx1 protein lacks antiviral activity against influenza A virus, as determined by viral protein expression and by an influenza virus minireplicon assay (5, 63). The importance of an intact GTPase domain was further demonstrated by the observation that human MxA interacts with THOV NP in a cosedimentation assay only in the presence of GTPγS. This nonhydrolyzable GTP analog pre-

sumably stabilized the MxA-NP interaction (105). It is not clear if GTPase activity is required or if GTP binding alone is sufficient for antiviral activity. GTP binding and GTPase activity are intimately connected, and there are no reports on Mx mutants that lack GTPase but still have GTP-binding activity. However, one mutant of human MxA (L612K) lacks detectable GTPase activity but has antiviral activity against THOV in infection and in a minireplicon system (111). Since the L612K mutation is located near the C terminus of MxA, far from the GTP-binding domain, this mutant can probably bind GTP, indicating that GTP binding is sufficient for the antiviral activity of MxA (111).

The activity of Mx proteins against orthomyxoviruses is neutralized by the 2C12 antibody (68, 71, 84, 112). This indicates a similar virus recognition region in three Mx homologs: MxA (residues 432 to 471), mouse Mx1, and rat Mx1. This 2C12 region is well conserved in all Mx proteins, which suggests that this region is important for its biological function. Besides the 2C12 epitope region, a second loop in the stalk domain of Mx proteins was recently found to be a genetic determinant of the species-specific antiviral activity of primate MxA proteins against members of the *Orthomyxoviridae* family (109). This loop, called L4 (MxA residues 533 to 572 [Fig. 1]), is essential for the antiviral activity of primate MxA against THOV and is also essential in the interaction between active MxA proteins and THOV NP protein. In addition, replacing loop L4 in mouse Mx1 with the human variant enhances its antiviral activity against THOV and allows the coimmunoprecipitation of THOV NP. However, replacing this loop in inactive primate MxA proteins by the active human MxA L4 loop is not enough to provide full protection against THOV infection. This suggests that other determinants are also important for MxA activity. Finally, loop L4, and residue F561 in particular, is essential for the antiviral activity of human MxA against influenza viruses, which means that loop L4 is important in the protection against orthomoxoviruses in general (109). This loop is less conserved than the 2C12 region. Particularly the second part of the loop, which contains residue F561, differs strongly among Mx proteins. It is tempting to speculate that this part of Mx is a determinant of its viral target specificity. The position of both loops at the tip of the Mx stalk suggests that this region is important for virus target recognition (Fig. 1B). This similarity in virus recognition provides support for the recently proposed model of a general antiviral activity against *Orthomyxoviridae*, in which MxA oligomerizes around incoming vRNPs and prevents their nuclear import (73, 104). However, subtle differences in Mx proteins, in particular their subcellular localization, are involved in antiviral specificity.

## Antiviral Activity against *Rhabdoviridae*

Vesicular stomatitis virus (VSV) is inhibited by most cytoplasmic Mx proteins (references are given in Table 1). MxA inhibits VSV mRNA synthesis, probably by affecting elongation of the viral RNA chain, and requires an intact GTPase domain (16, 63, 110). Inhibition of viral transcription was confirmed by an *in vitro* study using purified MxA and viral vRNPs (113). Purified MxA inhibits VSV transcription in the presence of GTP as well as in the presence of nonhydrolyzable GTP analogs (GMP-PNP or GTPγS) (69, 113). This suggests that GTP binding is sufficient for the antiviral activity of MxA against VSV and that GTPase activity is not needed. Although this *in vitro* study suggests that MxA targets the vRNPs of VSV, an interaction between human MxA and VSV proteins has not yet been demonstrated (16). The inhibition of

VSV mRNA synthesis resembles the inhibition of influenza virus transcription by mouse Mx1. However, it is not known if and how human MxA interferes with the interaction between the VSV nucleoprotein and the polymerase complex.

## Antiviral Activity against *Bunyaviridae*

Human MxA and mouse and rat Mx2 proteins inhibit different members of the *Bunyaviridae* (references are given in Table 1). The best-studied example is the inhibition of La Crosse virus (LACV) by human MxA (19, 23, 24, 27). MxA inhibits LACV replication by sequestering the newly produced viral nucleocapsid protein at large, membrane-associated perinuclear complexes. This missorting prevents the nucleocapsid protein from performing its function in viral replication (23, 27). In contrast, Frese and colleagues suggested that the active viral RNA polymerase complex is the target for MxA. They demonstrated that human MxA protein inhibits the accumulation of viral transcripts, and particularly the longer ones, suggesting an effect on elongation (19). However, this effect on elongation could be secondary to sequestration of the nucleocapsid protein. This sequestration would probably also affect mostly the large segments, as they need more nucleocapsid protein for their replication.

MxA also seems to inhibit Crimean-Congo hemorrhagic fever virus (CCHFV) by a similar mechanism (26), indicating that it has a similar mode of action against other viruses of this family, including Rift Valley fever virus (RVFV), Hantaan virus (HTNV), Puumala virus (PUUV), and Tula virus (TULV) (19, 20). However, an argument against a general antiviral mechanism is the observation that the nucleocapsid protein of Dugbe virus (DUGV) appears to avoid MxA activity and is not sequestered to the perinuclear region by human MxA (114).

## Antiviral Activity against *Paramyxoviridae*

Human MxA has antiviral activity against some viruses from the *Paramyxoviridae* family (17, 115). These antiviral activities are dependent on cell type and virus strain. For example, MxA can inhibit measles virus in the human mononuclear cell line U937 and in the glioblastoma cell line U87 but not in Vero or Hep-2 cells (17, 21, 115). Moreover, MxA can inhibit primary transcription in U87 cells but not in U937 cells (17), in which it inhibits viral glycoprotein synthesis instead (115). Human respiratory syncytial virus (HRSV) is not inhibited by MxA in U87 or Vero cells (116), but its close relative murine pneumovirus is inhibited by transgenic mouse cells expressing bovine (*Bos taurus*) Mx1 protein (117).

## Antiviral Activity against Other Viruses

The antiviral activities of the different Mx proteins described so far are all directed against negative-stranded RNA viruses. Nevertheless, human MxA and different fish Mx proteins have antiviral activity also against other RNA and even some DNA viruses. Human MxA has antiviral activity against the positive-stranded RNA viruses Semliki Forest virus (SFV) and classical swine fever virus (CSFV) (21, 24, 28). Also, Mx proteins of fish confer resistance against positive-stranded RNA viruses (32, 35, 37, 118). For example, grouper Mx can inhibit yellow grouper nervous necrosis virus (YGNNV) by inhibiting viral mRNA synthesis. The inhibition is likely mediated by the observed interaction between Mx and the viral coat protein, which probably disturbs the localization of the coat protein (32). In addition, the barramundi Mx

protein inhibits nervous necrosis virus (NNV) by redistribution of the viral RNA-dependent RNA polymerase to the perinuclear area, where it is degraded (37). Human MxA and fish Mx proteins can also inhibit double-stranded RNA (dsRNA) viruses. For example, salmon Mx and seabream Mx proteins can inhibit infectious pancreatic necrosis virus (IPNV) (30, 34, 119).

Mx proteins also have antiviral activity against dsDNA viruses. Human MxA can inhibit hepatitis B virus (HBV) (25, 120, 121) and African swine fever virus (ASFV) (29). MxA inhibits HBV replication at several steps during the viral life cycle. For example, MxA can inhibit the nucleocytoplasmic export of viral mRNAs, probably by being redistributed partly to the nucleus during HBV replication (25, 120). It was also demonstrated that MxA can interact with the hepatitis B virus core antigen, leading to its sequestration and immobilization in perinuclear compartments (121). This antiviral mechanism resembles the antiviral activity of MxA against LACV, an RNA virus (23, 27). Remarkably, inhibition of HBV by MxA is retained by MxA mutants with K83A or T103A substitutions. These mutations abolish the GTPase activity of MxA and are detrimental for the antiviral activity of MxA against RNA viruses (63, 120). This demonstrates that GTPase activity of MxA is dispensable for the inhibition of HBV (120). Finally, MxA protein complexes inhibit ASFV replication by encircling the virus factories (29).

## CONCLUSIONS

The studies described here collectively demonstrate that Mx proteins have a broad range of antiviral activity. It is unclear if all the sensitive viruses are inhibited by a common mechanism, but in general, Mx proteins recognize the nucleoproteins or (nucleo)-capsid proteins of different viruses, perhaps concurrently with the viral polymerase. The C termini of Mx proteins are responsible for recognition of the viral targets and for their differential antiviral activities. Identifying the common molecular pattern in the viral target proteins that is recognized by the Mx proteins is an exciting research objective. One possible common feature is the nucleotide-binding viral proteins. Moreover, the arrangement of multiple nucleoprotein or (nucleo)capsid protein molecules, e.g., in vRNPs or virus factories, allows extensive, repetitive contacts between Mx complexes and the viral targets. Consequently, even a weak-affinity contact could result in a biologically significant interaction.

Recent studies indicate ongoing coevolution between viruses and antiviral Mx proteins. For example, the stronger resistance of human influenza virus strains (relative to that of avian strains) against MxA activity illustrates the evolution of seasonal influenza virus strains to cope with MxA. This phenomenon is also seen for swine influenza viruses, although less prominently due to the lower antiviral activity of the swine Mx1 protein (95). The higher sensitivity of avian influenza virus strains suggests that MxA operates as an important barrier against the zoonotic introduction of avian influenza viruses in the human population. Understanding the molecular determinants of Mx resistance will help to estimate the pandemic potential of new influenza strains. In addition, a recent study also highlights the importance of this coevolution by revealing specific residues in primate MxA proteins which are under positive selection. These residues (mainly in loop L4) are predicted to be important in viral target recognition (109) (the evolution of MxA antiviral diversity was recently reviewed in reference 122).

How do Mx proteins perform their antiviral functions? An intact GTPase domain is required for activity against RNA viruses, but it is not clear if GTP binding is sufficient or GTPase activity is also needed. As the crystal structure of MxA is based on nucleotide-free MxA, it will be interesting to determine the conformational changes caused by GTP binding and GTP hydrolysis in an enzymatically active MxA protein. The information obtained from the crystal structure of MxA, together with mutational studies, led to the proposition of an antiviral mechanism that depends on the multimeric assembly of Mx proteins. In this model, Mx forms tetramers that further oligomerize into large Mx rings at higher Mx concentrations. These rings contain GTPase domains on the outside of the ring and stalks pointing inwards (123). This assembly and oligomerization allow the Mx stalk to interact with viral target structures, e.g., vRNPs, possibly leading to multiple Mx rings wrapping around these structures. Adjacent Mx rings can interact through their GTPase domains, leading to enhanced GTPase activity. This conformation allows cross talk between the GTPase domains and the stalks of neighboring Mx molecules and causes conformational changes leading to constriction of Mx rings and disruption of functional viral vRNPs by a mechano-chemical cycle. In this way, Mx rings could disrupt the association of nucleoprotein complexes with the viral polymerase, thereby inhibiting virus transcription or replication. These Mx rings could also act to relocalize viral (ribo)nucleoproteins or (nucleo)capsid proteins to perinuclear complexes in a GTPase-dependent way, leading to their sequestration and/or degradation. Although this ring-forming model is very appealing, there is no proof that ring formation of Mx around vRNPs or other viral structures actually occurs.

To summarize, Mx proteins are evolutionarily conserved, and they inhibit a wide range of viruses, probably by recognizing large viral structures possessing a degree of symmetry and/or iteration. In this sense, Mx proteins could be considered pattern recognition molecules with a direct antiviral effector function.

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