We have employed molecular genetic approaches to understand the domain organization of the HIV-1 resistance factor myxovirus resistance 2 (MX2). First, we describe an essential triple-arginine motif in the amino-terminal domain. Second, we demonstrate that this 91-residue domain mediates antiviral activity when appended to heterologous proteins, and we provide genetic evidence that protein oligomerization is required for MX2 function. These insights will facilitate future work aiming to elucidate MX2’s mechanism of action.

Type I interferons (IFNs) are produced in response to acute virus infection and evoke an antiviral state in susceptible cells through the expression of a diverse array of IFN-stimulated genes (ISGs) (1–3). Prominent among these are the dynamin-like guanosine triphosphatases (GTPases) MX1 (or MXA) and MX2 (or MXB) (4–6). The broad antiviral properties of MX1 have been recognized for many years, with the inhibitory effects on the orthomyxoviruses influenza A virus and Thogoto virus having been defined in detail (4, 5). In contrast, MX2, which previously had not been ascribed an antiviral function, was recently found to be a suppressor of primate immunodeficiency viruses, and particularly human immunodeficiency virus type 1 (HIV-1), but not of other retroviruses, such as murine leukemia virus (MLV) (7–9).

MX1 and MX2 each comprise an amino-terminal GTPase domain and a carboxy-terminal stalk domain that are connected by a tripartite bundle signaling element (BSE). A disordered loop (L4) in the stalk domain of MX1 interacts with orthomyxovirus nucleoproteins, and this is thought to promote the assembly of higher-order MX1 oligomers, GTPase activation and GTP hydrolysis, and conformational changes that disrupt viral replication complexes and their function (5, 10–12). MX2 antiviral function appears to be quite different: GTPase activity is dispensable (7, 9, 13), oligomerization beyond dimers appears unnecessary for viral suppression (14, 15), and antiviral specificity is determined by its amino-terminal 91 residues rather than L4 (13, 16, 17). Current models suggest that human MX2 recognizes the capsid (CA) lattice of HIV-1 reverse transcription complexes (RTCs) (7–9, 14, 17) and that inhibition occurs at a late posttranslational stage that is reflected as suppressed nuclear import and proviral formation (7, 9, 18).

As a step toward understanding the molecular basis for MX2 function, we sought to define the regions and residues of the amino-terminal 91 amino acids required for activity. Accordingly, scanning mutational changes that disrupt viral replication complexes and their function (5, 10–12). MX2 antiviral function appears to be quite different: GTPase activity is dispensable (7, 9, 13), oligomerization beyond dimers appears unnecessary for viral suppression (14, 15), and antiviral specificity is determined by its amino-terminal 91 residues rather than L4 (13, 16, 17). Current models suggest that human MX2 recognizes the capsid (CA) lattice of HIV-1 reverse transcription complexes (RTCs) (7–9, 14, 17) and that inhibition occurs at a late posttranslational stage that is reflected as suppressed nuclear import and proviral formation (7, 9, 18).

We next replaced arginines 11 to 13 individually with alanine (Fig. 1B). Although each residue appears to contribute to the full antiviral behavior of MX2, the arginines at positions 12 and 13 are more influential. We also assessed the importance of charge at these positions by replacing all three arginines with lysines. As with the triple-alanine substitution, this protein was nonfunctional, indicating that antiviral activity requires the presence of arginines at these positions rather than positively charged amino acids. Previous work has established that the transfer of the amino-terminal 91 amino acids of MX2 to MX1 confers a robust HIV-1-inhibitory phenotype (13). Consistent with the above observations, introduction of the RRR11–13A mutation into the MX1(NMx2) chimera also abrogated HIV-1 inhibition (Fig. 1C). Taken together, we conclude that the triple-arginine motif at positions 11 to 13 of human MX2 is essential for anti-HIV-1 function.
Indirect immunofluorescence analysis of transiently trans-
fected HeLa cells revealed that both wild-type MX2 and the
RRR11–13A mutant accumulated at the nuclear envelope (NE),
throughout the cytoplasm, and in large cytoplasmic bodies/gran-
ules (Fig. 2). Similarly, both the wild-type MX1(NMX2) chimera
and its RRR11–13A derivative also displayed similar patterns of
localization. These observations indicate that modified subcellu-
lar localization does not account for the lack of antiviral function
of RRR11–13A mutant MX proteins.

The canine MX2 ortholog does not inhibit HIV-1, but replace-
ment of the amino-terminal 29 amino acids of the canine protein
with those from the human counterpart creates a chimeric protein
as effective as human MX2 in suppressing HIV-1 infection (16).
Inspection of this region reveals 15 amino acid differences (Fig.
3A), with the critical arginine at position 13 being histidine in the
canine protein (indicated with an arrow). As expected, canine
MX2 does not exhibit an anti-HIV-1 phenotype in our system, but
we found that replacing this single histidine with arginine,
cmx2H13R, confers inhibitory function to a level close to that of
human MX2 (Fig. 3B). This finding further underscores the crit-
ical contribution of the triple-arginine motif to the anti-HIV-1
properties of MX2.

Given that transfer of the amino-terminal 91 amino acids of
MX2 onto MX1 bestows full anti-HIV-1 function (13), we wished
to determine whether this domain would still have this capability
when appended to an entirely unrelated protein. We initially
chose to use mouse Fv1b as the substrate since this protein is an
inhibitor of retrovirus infection (N-tropic NLV but not HIV-1)
(20,21), suppression is manifested as a lack of viral cDNA inte-
gration (22,23), and it is naturally oligomeric (24). Remarkably,
fusing residues 1 to 91 of MX2 to the amino terminus of Fv1b
(NMX2-Fv1b) conferred potent HIV-1-inhibitory activity, whereas
the parental Fv1b protein had no effect (Fig. 4A). Importantly, a
genetic constraint of MX2 function was faithfully preserved, as
introduction of the RRR11–13A mutation into this chimeric pro-
tein abrogated activity. As a confirmation of Fv1b functionality,
both fusion proteins suppressed N-MLV (but not B-tropic MLV)
infection as efficiently as the wild-type protein (Fig. 4B). There-

FIG 1 Definition of an essential triple-arginine motif in the amino-terminal domain of human MX2. (A) Alanine scanning mutagenesis of the amino-terminal domain
of MX2. (Upper panel) U87-MG/CD4/CXCR4 cells were transduced with EasiLV expressing CD8 (Neg Ctrl), TRIMCyp, MX2 short isoform (MX226-715), wild-type
(WT) MX2 without a Kozak sequence [MX226-715Kozak], or a series of WT and alanine scanning mutants (as indicated) bearing the Kozak sequence. The cells were treated
with doxycycline (0.5 μg/ml) for 72 h and challenged with an HIV-1-based lentiviral vector expressing GFP (HIV-1/GFP) at a multiplicity of infection of 0.2. The percentage of GFP-expressing cells was evaluated by flow cytometry 2 days later. Mean percentages of transduced cells from three independent experiments are shown.
(Lower panel) Immunoblot analysis of parallel samples from upper panel. Protein levels of Flag-tagged MX2 proteins were determined, and Hsp90 served as a loading
control. (B) Effect of individual-alanine and triple-lysine mutations in the triple-arginine motif. U87-MG/CD4/CXCR4 cells expressing CD8 (Neg Ctrl), MX226-715,
WT, or Kozak sequence-bearing WT MX2, or single-alanine or triple-lysine MX2 mutations were challenged with HIV-1/GFP vector at a multiplicity of infection of
0.2, and infection was analyzed by flow cytometry 2 days later, as in panel A. Mean percentages of transduced cells from three independent experiments are shown.
(Lower panel) Immunoblot analysis of parallel samples from upper panel was performed as for panel A. (C) Effect of disrupting the triple-arginine motif in the context
of MX1(NMX2) chimera. U87-MG/CD4/CXCR4 cells expressing CD8 (Neg Ctrl), MX226-715, WT MX1(NMX2), and the RRR11–13A mutant were challenged with HIV-1/
GFP at a multiplicity of infection of 0.2. Mean percentages of transduced cells from four independent experiments are shown. (Lower panel) Immunoblot analysis of
parallel samples from upper panel was performed as for panel A.
fore, the amino-terminal domain of human MX2 is the only element of MX1/MX2 that is necessary for HIV-1 inhibition in the context of a heterologous fusion partner.

In a final series of experiments, we turned to a system where the oligomeric status of the MX2 fusion partner can be experimentally manipulated: namely, the well-characterized leucine zipper domain of yeast GCN4 (25). We therefore placed residues 1 to 91 of MX2 at the amino terminus of monomeric, dimeric, and trimeric versions of this leucine zipper (25, 26). As shown in Fig. 4C, the fusions containing the dimeric or trimeric zippers each suppressed HIV-1 by ~80%, whereas the monomeric protein had no effect. In sum, the amino terminus of MX2 is sufficient to inhibit HIV-1 infection, provided that it is contained within a protein that can oligomerize, at least into dimers.

Here, we have employed site-directed mutagenesis to define an essential triple-arginine motif within the amino-terminal domain of human MX2 (Fig. 1 and 3), findings that are consistent with analyses of deletion mutants (17, 18). Previous work has revealed that residue 37 can also play a role in suppression mediated by the MX2 protein of macaque (16), but our alanine scanning failed to register the importance of this amino acid. Importantly, the triple-arginine motif is required for HIV-1 suppression irrespective of the scaffold to which the amino-terminal domain is attached, indicative of a vital role in MX2 function (Fig. 1 and 4).

A central question for unraveling the molecular basis for MX2 action is, therefore, identification of the relevant interacting partners for this element. The most likely possibilities are the RTC CA lattice and/or cellular factors. Although interactions between MX2 and viral cores or in vitro-assembled CA-containing nanotubes have been reported, their relevance for the inhibition of infection is currently uncertain, as they do not conform to the genetic determinants of HIV-1 suppression in that MX2-resistant CA proteins still interact with MX2 (14, 17). Future work will also explore potential interactions with host factors. Given that MX2’s amino-terminal domain is sufficient to confer NE localization (13) and nuclear localization signals are frequently rich in lysines and/or arginines, the finding that the RRR11–13A protein displays the same staining pattern as that of the wild-type protein (Fig. 2) was unexpected and deserves further attention. Either way, future interrogation of these possibilities will be important.
FIG 3 A histidine-to-arginine alteration at position 13 in canine MX2 confers potent anti-HIV-1 function. (A) ClustalW sequence alignment of the first 91 amino acids of human and canine MX2. Asterisks, identical amino acids; colons, conserved substitutions; periods, semiconserved substitutions. (B) U87-MG/CD4/CXCR4 cells were transduced with EasiLV expressing CD8 (Neg Ctrl) or amino-terminally Flag-tagged human MX2 (huMX2), canine MX2 (caMX2), or canine MX2H13R. The cells were treated with doxycycline for 72 h and challenged with HIV-1/GFP at a multiplicity of infection of 0.25. The percentage of GFP-expressing cells was evaluated by flow cytometry 2 days later. Mean percentages of transduced cells from three independent experiments are shown. (Lower panel) Immunoblot analysis of parallel samples from the upper panel was performed as for Fig. 1A. Of note, the amino-terminal Flag prevents leaky scanning, resulting in exclusive production of the long isoform of MX2.

FIG 4 The amino-terminal domain of MX2 inhibits HIV-1 infection when transferred to heterologous scaffolds. (A) Fusion of the amino-terminal domain of MX2 to the amino terminus of Fv1\(^{b}\). U87-MG/CD4/CXCR4 cells were transduced with EasiLV expressing CD8 (Neg Ctrl), MX2, or amino-terminally Flag-tagged Fv1\(^{b}\), N\(_{MX22-RRR11-13A}\)-Fv1\(^{b}\); treated with doxycycline for 3 days; and challenged with HIV-1/GFP. The percentage of infected cells was analyzed 2 days later by flow cytometry. Mean percentages of transduced cells from four independent experiments are shown. (Lower panel) Immunoblot analysis of parallel samples from the upper panel was performed as for Fig. 1A. (B) The N\(_{MX22-RRR11-13A}\) fusions inhibit infection by N-MLV. 293T cells were transduced with EasiLV expressing CD8 (Neg Ctrl), Fv1\(^{b}\), N\(_{MX22-RRR11-13A}\)-Fv1\(^{b}\), and treated with doxycycline (2.5 \(\mu\)g/ml) for 3 days. The cells were then challenged with similar volumes (4 \(\mu\)l) of GFP-encoding N-MLV and B-MLV viral stocks (produced by 293T cotransfection of pMD.G, p13077, and pCIG3N or pCIG3B expression plasmids, respectively) (21, 27), and infection efficiency was analyzed by flow cytometry 2 days later. Mean percentages of transduced cells from three independent experiments are shown. (C) Dimeric and trimeric leucine zipper fusion proteins containing the amino-terminal domain of MX2. U87-MG/CD4/CXCR4 cells were transduced with EasiLV expressing CD8 (Neg Ctrl), MX2, and carboxy-terminally Flag-tagged fusion proteins containing the amino-terminal domain of MX2 grafted to different versions of the GCN4 leucine zipper, forming either dimers, trimers, or monomers (N\(_{MX22-RRR11-13A}\)-LZdim, N\(_{MX22-RRR11-13A}\)-LZtrim, and N\(_{MX22-RRR11-13A}\)-LZmon, respectively); treated with doxycycline for 3 days; and challenged with HIV-1/GFP. The percentage of infected cells was analyzed 2 days later by flow cytometry. Mean percentages of transduced cells from three independent experiments are shown. (Lower panel) Immunoblot analysis of parallel samples from the upper panel was performed as for Fig. 1A.
Our results with MX2 fusions to leucine zippers imply the importance of protein dimerization for HIV-1 inhibition (Fig. 4). This conclusion corroborates recent mutagenesis studies performed in the context of full-length MX2, which indicate that dimerization, but not higher-order oligomerization, is necessary for antiviral function (14, 15). This feature of MX2 therefore appears to be another significant departure from the current models for MX1-mediated viral suppression (5, 10). Further work will be needed to understand the differing oligomerization requirements of the MX proteins for the inhibition of distinct viral families.

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