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An evolutionary perspective on the broad antiviral specificity of MxA

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Abstract

Germ line encoded antiviral defenses in vertebrate cells tend to be either broadly acting factors that exploit general features of viral replication or effectors with strong pathogen preference by virtue of specific recognition of viral proteins. The Mx GTPases, however, are atypical since they have broad antiviral activity against a wide range of RNA and DNA viruses despite specifically targeting different proteins across virus families. This review presents recent advances in understanding the biochemical properties and evolution of the primate ortholog MxA, and discusses how this information begins to provide molecular insights into the mechanisms behind the intriguing conundrum of how MxA is able to engage a diversity of viral proteins yet elicit antiviral breadth.

Introduction

In 1962, a short report described the unique resistance of the inbred mouse strain A2G to mouse-adapted influenza virus [1]. This observation led to mapping of myxovirus resistance 1 gene (*Mx1*, encoding Mx1 in mice and MxA in humans), which provides cell intrinsic defense against viral infection following induction by interferon (IFN) signaling. Constitutive expression of human MxA was sufficient to confer resistance to viral infection in *Mx1* and IFN receptor α/β null mice [2]. Several studies have underscored the major phenotypic effect of MxA as a remarkably broad-acting single gene effector of innate immunity against viruses (reviewed in [3]).

There exists an apparent dichotomy among the intracellular antiviral defenses; they either act broadly by virtue of recognizing general cues of viral infection (Figure 1A, blue), or are specific to a particular virus by virtue of highly specific recognition of viral components (Figure 1A, yellow). However, the Mx GTPases are atypical because they act broadly against a wide-spectrum of RNA and DNA viruses [3] via highly specific recognition of

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different viral proteins in diverse viruses (Figure 1A, green). How MxA combines target specificity with antiviral breadth is largely unknown. The recent confluence of biochemical, structural and evolutionary studies has begun to provide key insights into resolving the apparent paradox of MxA antiviral breadth in spite of target specificity. Here, we discuss the repertoire of MxA-sensitive viruses, how MxA influences virus evolution and how viruses, in turn, might affect MxA function and evolution.

Viral targets of MxA

The molecular interactions between MxA and viral targets have been most fully described for the *orthomyxoviruses*, and in particular for influenza A virus. Multiple lines of evidence suggest that NP is the primary viral protein underlying susceptibility to MxA. For example, the differential susceptibility of avian H5N1 (susceptible) and human H1N1 (resistant) influenza viruses to Mx1 is determined solely by the NP segment [4]. MxA also interacts with the NP protein from the tick-borne *orthomyxovirus* Thogotovirus (THOV) [5]. A recent study mapped the basis of MxA escape by the 1918 and 2009 H1N1 pandemic strains to changes in the NP [6]. These escape residues, which are clustered on the NP surface, are sufficient to confer MxA-resistance when introduced into the otherwise susceptible H5N1 NP. Thus, the NP protein is necessary and sufficient to determine MxA susceptibility in *orthomyxoviruses*.

Recent studies have revealed that the influence of MxA stretches far beyond *orthomyxoviruses*. Indeed, the breadth of MxA antiviral activity encompasses a striking diversity of both negative- and positive-sense RNA viruses, including *bunyaviruses*, *paramyxoviruses*, *picornaviruses*, *rhabdoviruses* and *togaviruses* (reviewed in [3]). Although the nature of MxA interactions with other viruses has not been resolved to the same detail as with *orthomyxoviruses*, a nonetheless amazing diversity of viral targets has emerged. For example, infection by *Bunyaviridae* results in co-localization of MxA and the viral nucleocapsid (N) protein at an ill-defined perinuclear compartment [7], which in turn blocks viral replication. This suggests that similarly to the *orthomyxovirus* NP, MxA engages the *bunyavirus* N protein to elicit its antiviral function. MxA also restricts Semliki Forest virus (SFV), a positive-sense RNA virus [2,8]. This restriction appears to be independent of SFV structural proteins, suggesting that MxA targets a component of the SFV replicase [8]. Therefore, MxA may target proteins with similar functionality across divergent viral life cycles. However, the existence of a pan-viral epitope for MxA targeting is difficult to reconcile with recent studies that extend MxA antiviral activity to DNA viruses. For example, MxA inhibits the *hepadnavirus* hepatitis B virus (HBV) [9] via the hepatitis B core antigen protein (HBcAg) [10]. MxA has also been reported to restrict large double-stranded DNA viruses including the *orthopoxvirus* monkeypox [11] and the poxvirus-like *Asfarvirus* African swine fever virus (ASFV) [12]; for these viruses MxA targets are unknown. Taken together, these studies show that MxA interacts with highly divergent proteins across a diversity of viral families. This provides a molecular dilemma for MxA target recognition: how does MxA maintain the ability to recognize so many different viral proteins, each with the capacity to rapidly evolve to evade recognition?

Mechanism of MxA action

The *Mx1* gene encodes a protein comprised of an amino (N)-terminal GTPase domain, middle domain and carboxy (C)-terminal GTPase effector domain (GED) [13]. Phylogenetically, MxA proteins are most closely related to Dynamin and Dynamin-like GTPases [14]. As such, MxA exhibits canonical Dynamin-like characteristics of low affinity for guanine nucleotides and high intrinsic rates of GTP hydrolysis, which is dose-responsive

and dependent on oligomerization [15]. However, an understanding of how the GTPase function contributes to MxA antiviral activity has been elusive.

Two crystal structures of the human MxA protein [15,16] reveal an elongated three-domain protein comprised of an N-terminal globular GTPase-containing head (G domain) and a C-terminal helical stalk, which are connected by a hinge-like bundle-signaling element (BSE) (Figure 2A). The asymmetric unit revealed a domain-swapped orientation of two MxA monomers, permitting the identification of intermolecular contacts in the stalk and BSE domains that, when mutated, abolish tetramerization and higher order structure. Although MxA exists as a stable tetramer in solution, cryo-electron microscopy of both Dynamin [17] and MxA [18] indicate significant higher-order oligomerization. Molecular modeling of MxA oligomers predicts a stalk-mediated assembly into a ring-shaped antiviral complex, with G domains facing outward [16]. Importantly, this model provides an explanation for oligomerization-dependent GTP hydrolysis, where self-propagating GTPase activity is dependent on higher-order assembly that brings G domains of neighboring tetramers in close proximity [19–21] (Figure 2B). Moreover, this orientation suggests that upon formation of the MxA antiviral complex GTP binding and hydrolysis can coordinately signal through the BSE to the stalk, resulting in conformational changes that may elicit mechanoenzymatic force on targeted structures, similar to that of Dynamin reorganization of associated membranes [17].

Although the MxA structures suggest a compelling model for how the basic GTPase protein architecture elicits antiviral activity, the structural studies also highlight difficulties in understanding MxA target recognition. The structures reveal, for instance, that multiple protein-protein contacts govern GTP hydrolysis and oligomerization. Therefore, mutagenesis or truncation-based methods have failed to decipher target recognition. For example, established Mx mutants that lack antiviral activity (e.g., H630K in rat Mx2) were revealed to do so because of disruption of intramolecular contacts between the BSE and stalk [16,22]. Thus, although the structural analyses nicely elucidate the nature of the MxA antiviral complex, they still leave unanswered the important question of how this complex might engage with its myriad viral targets.

MxA and viral evolution reveal target specificity determinants

To understand MxA target recognition, we recently used an alternative approach that leverages the evolutionary history of host-virus interactions [23]. Interactions between viral proteins and intracellular defenses represent key molecular battlegrounds that significantly influence host resistance or susceptibility. Because successful engagement by one party comes at the detriment of the other, host virus protein-protein interactions rapidly evolve to establish or evade recognition (Figure 3A). We can use signatures of rapid evolution to gain substantial molecular insights into host-virus interactions. Rapid evolution can be formalized by the dN/dS statistic, which calculates the observed rate of non-synonymous (dN) relative to synonymous (dS) nucleotide substitutions in an alignment of orthogonal sequences. dN/dS ratios higher than one are indicative of positive selection, whether calculated as an average over the entire gene or on a per codon basis. These “hotspots” of positive selection predict residues that significantly impact the affinity of host-viral interactions [24]. Such studies have identified key domains/residues that determine either escape of some host antiviral proteins from viral antagonism or those that allow host proteins to recognize altered viral epitopes to maintain their antiviral function (reviewed in [24,25]).

The broad antiviral activity of MxA suggests that it has been involved in multiple arms race conflicts throughout its history (Figure 3B). Such a conflict-ridden past would be predicted to result in particularly strong evolutionary signatures of positive selection in MxA.

Moreover, since MxA manifests its antiviral action via specific recognition of viral epitopes, "hotspots" of positive selection could therefore be used to directly identify at least some of the key determinants of MxA target recognition. In an analysis of MxA orthologs from simian primates, we identified the disordered loop L4, which protrudes from the stalk domain (Figure 2A and 2B), as one such "hotspot" of positive selection. We found that variation in L4 explains differences in antiviral activity among primate MxA orthologs against THOV and influenza A viruses (FLUAV). Human L4 grafted onto mouse Mx1 conferred both gain of antiviral activity and concomitant binding of Mx1 to the THOV NP. Intriguingly, MxA antiviral specificity for THOV is largely governed by a single amino acid F561 in L4, which has been recurrently mutated throughout primate MxA evolution. Thus, the loop L4 is a key determinant of MxA interaction with NP proteins from *orthomyxoviruses*.

Although the large phenotypic effect of a single amino acid change in MxA seems extraordinary, other studies on the evolutionary dynamics between host antiviral genes and viruses have also uncovered occurrences of positively selected (adaptive) single residue changes with profound impacts on host-virus interactions (for example, [26–29]). The MxA structure, in combination with the evolutionary analysis, may also point to how single amino acid changes can have large functional outcomes. As described above, molecular modeling predicts that MxA oligomers form a ring-like complex. Interestingly, the loop L4 is positioned inward from the ring's inner surface (Figure 2B), suggesting that in its oligomeric state small changes elicited by single amino acids may act cooperatively to produce significant effects in viral target binding and consequently antiviral activity. Interestingly, another restriction factor TRIMCyp recognizes markedly different viral epitopes by interconverting a disordered surface loop between multiple conformations [30]. A similar mechanism may also afford MxA the flexibility to recognize divergent targets by virtue of the disordered nature of L4. MxA oligomerization and L4 flexibility may help explain how single residue changes in MxA L4 manifest such large effect phenotypes.

The specificity encoded by individual amino acids in MxA L4 provides a model to explain MxA recognition of multiple targets. Not only does the residue at 561 determine MxA antiviral specificity for THOV, but this specificity is also unperturbed by changes at distal or even neighboring amino acids that are also evolving under positive selection. This is intriguing since the MxA target interface is likely much broader than a single residue. Nonetheless, this finding does suggest a means by which MxA might maintain binding specificities to multiple targets. For example, whereas residue 561 may specify MxA specificity for THOV, other "hotspots" may coincide with distinct pathogen preferences that are independently specified. Moreover, other positively selected sites outside L4 may similarly act as specificity determinants for other viruses [24]. Indeed, we predict that differences in MxA antiviral activity against non-*orthomyxoviruses* could map outside L4.

The antiviral utility might be expected to be short-lived for proteins like MxA that recognize a specific target against rapidly evolving viruses. Insight into how MxA potentially circumvents this problem comes from the recent mapping of Mx-resistance residues on the influenza virus NP [6]. Surface exposed residues were identified in the NP of human influenza viruses that were necessary and sufficient to confer protection against an Mx-sensitive avian H5N1 virus. Importantly, the number of residues required to gain Mx-resistance varied from 10 to four depending on whether amino acids were derived from the 2009 or 1918 pandemic H1N1 influenza virus strains. However, introducing MxA resistant mutations in the NP of avian H5N1 viruses caused significant attenuation of viral growth in the absence of MxA. Although this result is consistent with the more recent avian origin of the 1918 virus, it also highlights the possibility that several changes in the NP and the viral polymerase are required to epistatically compensate for compromised function in NP

proteins that acquired MxA-resistance. Therefore, MxA evasion and efficient replication may independently shape the viral fitness landscape that constrains NP evolution and influences influenza virus host range. These studies suggest that the fitness cost to the virus that results from MxA evasion may narrow the gap in 'evolvability' between host and viral proteins (Figure 3C). It is possible that natural selection has honed MxA recognition onto those surfaces of the virus that are evolutionarily constrained by, for example, epistatic interactions. A similar strategy is utilized by viral antagonists that mimic highly constrained host proteins to subvert cellular processes [31]. In this context, virus adaptation in an intermediate host, in which the NP protein is not under tight surveillance by MxA, may represent an important evolutionary transition stage in allowing enough NP variation to overcome MxA restriction via a single evolutionary transition (Figure 3D).

Avenues of future exploration into MxA biology

Although the past few years have yielded much insight into MxA biology, many questions remain unanswered. For instance, the target for the majority of MxA-sensitive viruses is unknown. Experimental evolution schemes may help identify additional targets by selecting for escape variants on cells that are resistant to infection by virtue of MxA expression. Moreover, the description of residues that have been adaptively selected across primate MxA orthologs provides new tools to examine human MxA-resistant viruses. Further characterization of these residues may inform rationalized design of optimized MxA recognition surfaces. These insights in combination with cataloging of novel MxA targets may help to elucidate co-crystal structures between MxA and target proteins. We envision that the confluence of these strategies will provide a powerful model for how MxA alters target recognition in biochemical space and evolutionary time.

Genetic innovation in Mx proteins may also extend beyond positive selection at viral target interaction surfaces. For instance, rodent Mx paralogs have subfunctionalized wherein Mx1 localizes to the nucleus while the recently diverged Mx2 resides in the cytoplasm. These differences in cellular localization have led to the evolution of specificity toward viruses that replicate in the respective cellular niche of each paralog [3,32]. In this way, rodents have split the burden of antiviral breadth by subcellular compartmentalization of two active antiviral Mx proteins. Therefore, modification of MxA localization and membrane targeting may represent an evolutionary strategy to optimize the likelihood of contacts with viral targets. Akin to evolutionary insights into MxA target recognition, a detailed cytological survey of MxA orthologs may uncover more widespread modification of MxA localization. The capacity for rapid evolution at molecular interfaces is clearly one strategy to overcome the challenge of viral diversity; however, a combination of traits primed for adaptation, such as subcellular localization, may ultimately explain the antiviral breadth of MxA.

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Highlights

- MxA is an unusual antiviral defense protein because it is both broad and specific
- MxA structure clarifies the role of the GTPase architecture in antiviral function
- MxA and viral target proteins are locked in a molecular arms race
- Several rapidly evolving surfaces may underlie the broad antiviral range of MxA
- Evolution of MxA resistance in targeted viral proteins may incur a fitness cost

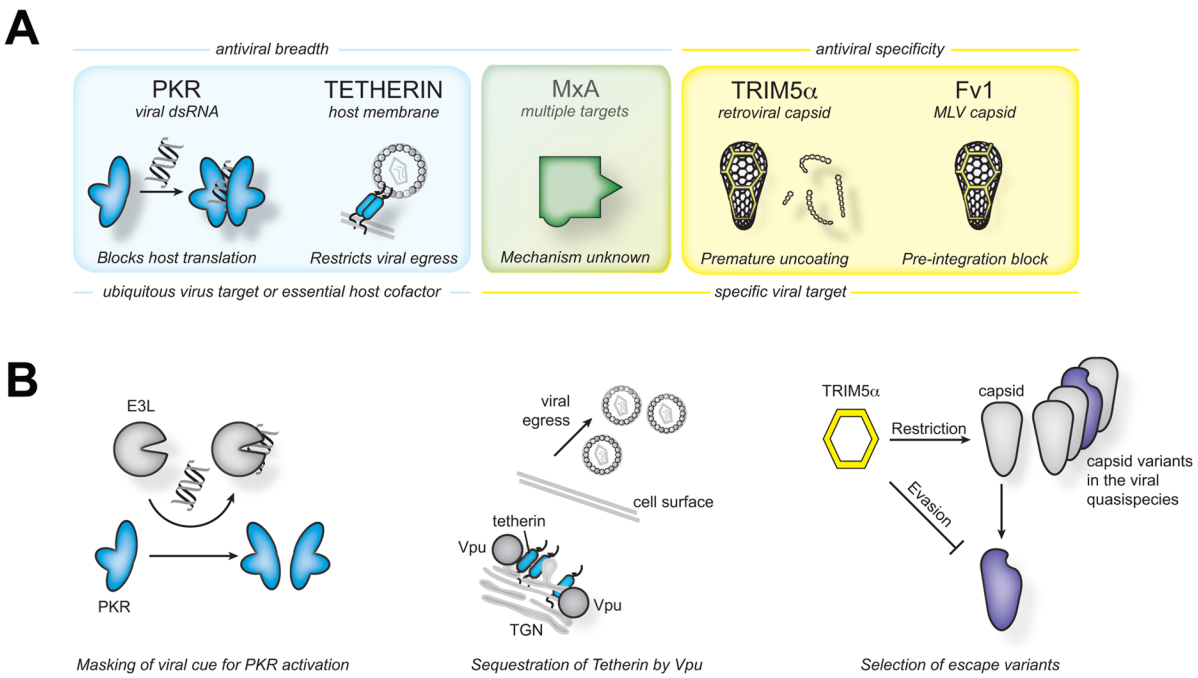

Figure 1. Target recognition determines the range in antiviral activity

A. Depicted are illustrative examples of broad (shown in blue) and narrow (shown in yellow) acting antiviral proteins. Broad acting antiviral factors tend to act through recognition of ubiquitous viral substrates. For example, double-stranded RNA sensing activates Protein kinase R (PKR), which blocks host protein synthesis through the phosphorylation of eIF2alpha [33]. Broad-acting antiviral factors can also act by targeting cellular substrates that are essential for viral replication. For example, tetherin incorporates into host membranes, effectively 'tethering' budding virions to the cell surface [34]. In contrast, the recognition of specific substrates narrows antiviral specificity. TRIM5alpha binds the retroviral capsid lattice, which promotes premature uncoating [35]. In turn, TRIM5alpha shows strong specificity for simian retroviruses. Similarly, murine Fv1 restricts B-tropic murine leukemia viruses (B-MLV) with exquisite specificity, which limits its activity against even the highly related N-tropic MLV [36] [37]. MxA is shown as a blend of these classification schemes (green), achieving antiviral breadth through recognition of distinct viral targets that vary across viral families.

B. Common viral evasion strategies in response to broad or narrow acting antiviral factors. Viruses often encode antagonists that either directly or indirectly overcome broad-acting antiviral factors. For example, the poxvirus-encoded antagonist E3L masks viral dsRNA thereby preventing PKR activation (left). Alternatively, HIV-1 Vpu sequesters tetherin at the transgolgi network (TGN) preventing its trafficking to the plasma membrane (middle) (reviewed in [25,38]). Host factors with specific targets can be overcome by selection of viral variants that carry escape mutations (right).

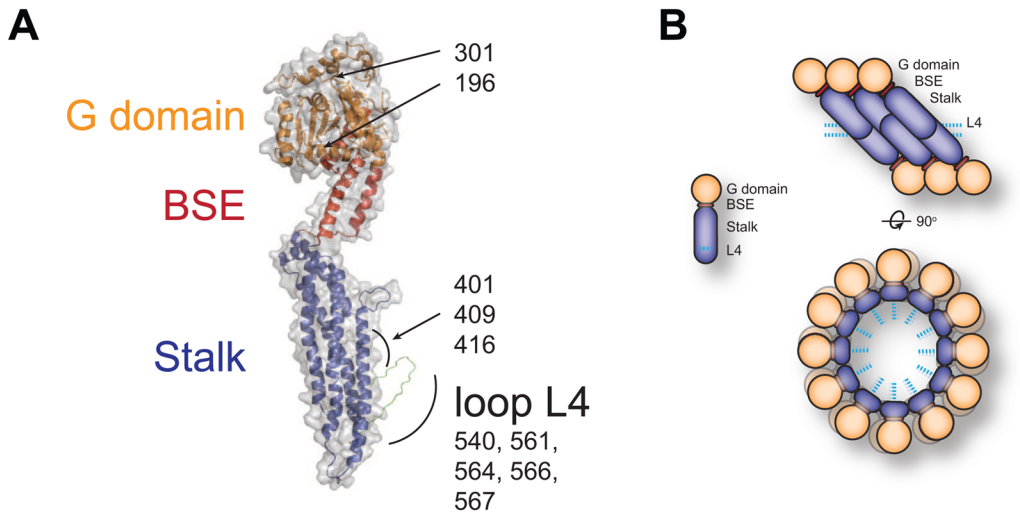

Figure 2. Structural and evolutionary insights into MxA antiviral activity

A. The crystal structure of human MxA (pdb 3SZR) [16] is depicted with the GTPase domain (G, orange), bundle-signaling element (BSE, red) and stalk (blue) oriented top to bottom. Sites found to be evolving under positive selection are indicated on the structure. Residue number refers to position in human MxA. The loop L4 "hot spot" (green, non-surfaced), which contains a patch of rapidly evolving residues, has been manually drawn in using the software PyMol [39].

B. Two illustrations of the MxA antiviral complex viewed from either the side or above. Oligomers assemble primarily through contacts in the stalk and BSE, such that GTPase domains face outwards positioning the loop L4 on the inner surface.

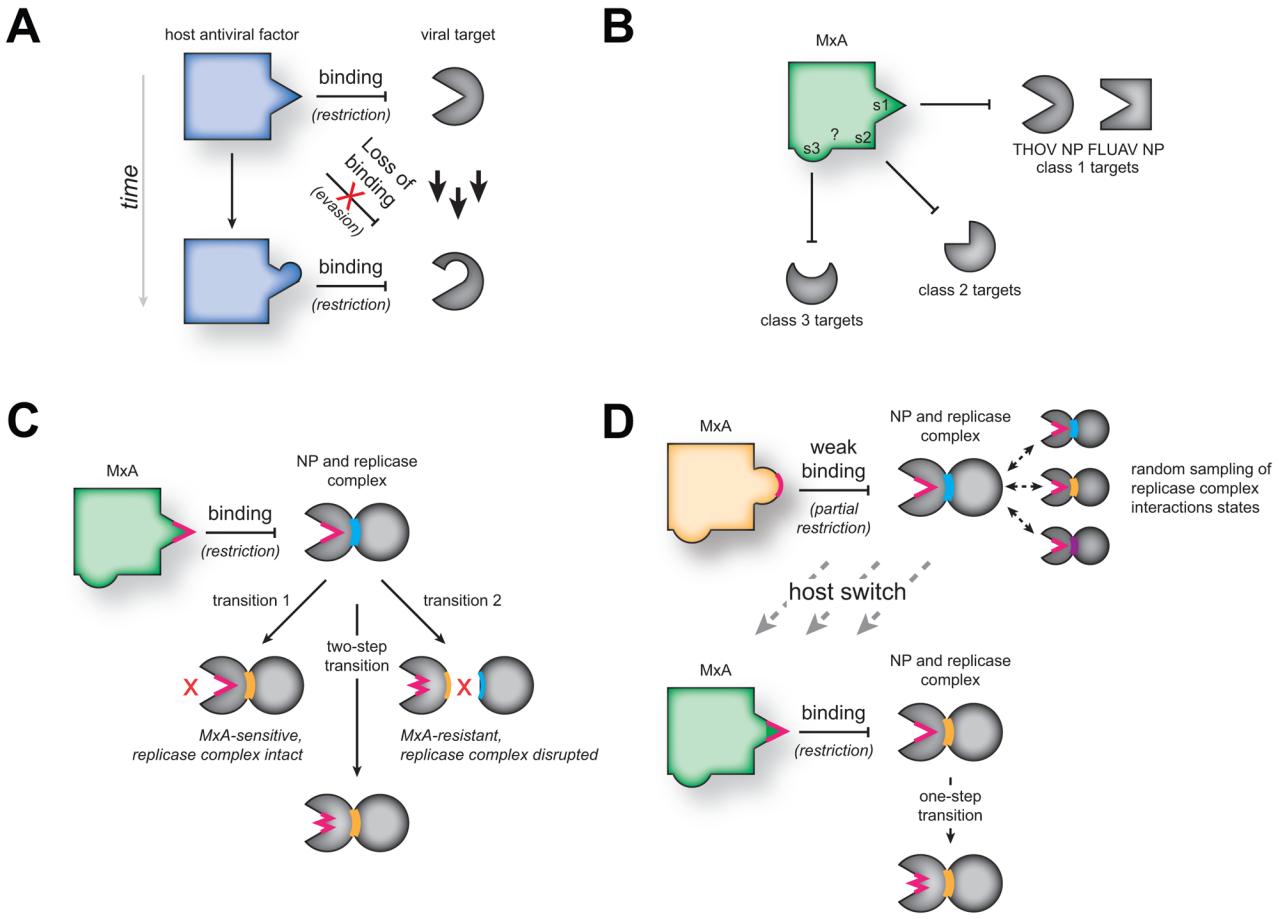

Figure 3. An arms race between MxA and targeted viral proteins

A. We depict a generalized arms race between a host antiviral factor (blue) and its viral target (grey). Host recognition, which restricts viral replication, selects for variants in the viral population that evade host factor binding. This in turn selects for host variants that re-establish target interaction. The effective population size and mutation rate of viruses decreases the number of generations required to evolve adaptive mutations (multiple, thick arrows). The 'direction' of the arms race can be reversed (e.g., viral antagonists that target broadly-acting antiviral factors; see Figure 1B). Note that host recognition can be driven both by selection of resistant viral variants or newly introduced pathogens (not shown).

B. The broad-acting antiviral protein MxA (green) is rapidly evolving under strong positive selection as a result of arms races that recurrently play out over evolutionary time against the diversity of MxA targets. Multiple 'hotspots' of positive selection on the MxA protein represent likely target interaction surfaces (illustrated as surface s1, s2 and s3). One experimentally validated target interaction site (L4, surface s1) governs MxA specificity against multiple *orthomyxoviruses*. Similarly, we speculate that other MxA surfaces might dictate specificity for distinct classes of viral targets.

C. MxA (green) may selectively target a viral surface on the influenza virus NP (grey) that is highly constrained for other functions. In the schematic, the evolution of NP is constrained both by its interaction with MxA (highlighted in pink) as well as the maintenance of a functional viral replicase [6], each representing a distinct fitness requirement. At the initial 'state' the viral population has reached local fitness peaks that satisfy both landscapes. Selective pressure from MxA forces the viral quasispecies off local optima to explore fitness valleys that represent significant barriers to sampling mutually fit

solutions. One adaptive outcome for NP might be to require mutations that modify the initial NP/replicase interaction (highlighted in blue) to maintain a functional NP/replicase (highlighted in orange) but also permits evasion of MxA. The model is agnostic as to whether mutations that modify the NP/replicase interface are restricted to the NP or also occur in other components of the viral replicase complex.

D. Virus adaptation to MxA in an intermediate host (orange) may represent an important evolutionary step in the chain of transmission for influenza and other viruses. Here, MxA binding to NP is weak such that the viral population is free to sample numerous NP/replicase 'states' (dashed lines, with sampled 'states' highlighted in blue, purple and orange). Upon transmission of the virus to a new host (green), a 'state' that permits evasion of MxA while maintaining NP/replicase interactions has been previously sampled, such that MxA can be overcome via a single evolutionary transition.