Structural Requirements for the Antiviral Activity of the Human MxA Protein against Thogoto and Influenza A Virus*

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Background: Interferon-induced human MxA GTPase is an innate immunity factor with broad antiviral activity.
Results: Antiviral specificity against orthomyxoviruses (influenza A and Thogoto viruses) is determined by a bipartite element in disordered loop L4.
Conclusion: A few critical amino acids allow multiple antiviral conformations of L4.
Significance: The MxA antiviral interface may provide clues for the design of novel antivirals.

The interferon-induced dynamin-like MxA protein has broad antiviral activity against many viruses, including orthomyxoviruses such as influenza A and Thogoto virus and bunyaviruses such as La Crosse virus. MxA consists of an N-terminal globular GTPase domain, a connecting bundle signaling element, and the C-terminal stalk that mediates oligomerization and antiviral specificity. We previously reported that the disordered loop L4 that protrudes from the compact stalk is a key determinant of antiviral specificity against influenza A and Thogoto virus. However, the role of individual amino acids for viral target recognition remained largely undefined. By mutational analyses, we identified two regions in the C-terminal part of L4 that contribute to an antiviral interface. Mutations in the proximal motif, at positions 561 and 562, abolished antiviral activity against orthomyxoviruses but not bunyaviruses. In contrast, mutations in the distal motif, around position 577, abolished antiviral activity against both viruses. These results indicate that at least two structural elements in L4 are responsible for antiviral activity and that the proximal motif determines specificity for orthomyxoviruses, whereas the distal sequence serves a conserved structural function.

The sensitivity of influenza A viruses (FLUAV) to MxA is determined by the viral nucleoprotein (NP), which is the main structural component of influenza virus nucleocapsids (6, 7). Nucleocapsids contain the viral genomic RNA in association with the viral polymerase and NPs and transcribe and replicate the viral genome in the cell nucleus. A physical interaction of MxA with the nucleocapsids of Thogoto virus (THOV, a tick-transmitted influenza-like orthomyxovirus) was demonstrated (8) and was shown to prevent the intracellular transport of the nucleocapsids into the cell nucleus, blocking viral transcription and replication (9). Accordingly, MxA seems to block FLUAV by interfering with nuclear import of incoming nucleocapsids in FLUAV-infected cells (10, 11) and by affecting later steps of viral replication (12). In the case of bunyaviruses, MxA tightly interacts with the viral nucleoprotein (N) and sequesters N protein in perinuclear complexes (13). Formation of MxA-N aggregates in infected cells is considered to be the basis of the antiviral effect (14).

The crystal structure of MxA reveals a three-domain architecture (15, 16). The N-terminal globular G domain is connected to an elongated stalk, consisting of a four α-helix bundle, by the bundle signaling element that is formed by three α-helices from the flanking regions of the G domain and the very C-terminal part of the molecule (see Fig. 1A). The G domain binds and hydrolyzes GTP, whereas the bundle signaling element is thought to transfer structural changes induced by GTP binding and hydrolysis to the rigid stalk. The stalk mediates self-assembly into large oligomers and provides antiviral effector functions. MxA self-assembles in a criss-cross fashion into extended multimers via interfaces formed between the stalks and bundle signaling elements of neighboring molecules (15, 16). The oligomers are detectable by electron microscopy and appear to form ring-like structures (17–19). The G domains are directed to the outer side of the multimeric rings, whereas the stalks are directed to the opposite inner side. A disordered loop of 40 amino acids, called L4, protrudes from the stalk domain and provides a key interface for the nucleocapsid proteins of

Human MxA is an innate antiviral protein belonging to the dynamin superfamily of large GTPases (1). It has intrinsic antiviral activity against many different viruses, including orthomyxoviruses and bunyaviruses, and its expression is strictly controlled by type I (α/β) or type III (λ) interferons (1). MxA accumulates in the cytoplasm and recognizes the nucleocapsids of invading viruses, leading to an early block of the viral replication cycle. Most recently, the human paralog MxB has been described as a comparable restriction factor for HIV-1. MxB interacts with the nucleocapsids of HIV-1 and appears to block the lentiviral replication cycle at a step after reverse transcription and before chromosomal integration (2–5).

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The abbreviations used are: MxA, human MxA; FLUAV, influenza A virus; hsL4, Homo sapiens loop L4; LACV, La Crosse virus; mNL4, Mus musculus loop L4; MOI, multiplicity of infection; Mx1, murine Mx1; Mx, myxovirus resistance; N, nucleoprotein of LACV, NP, nucleoprotein of THOV and FLUAV; THOV, Thogoto virus.

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orthomyxoviruses. MxA self-assembly is required for antiviral activity (15, 16), most likely because the oligomer allows multiple contacts of L4 with the repetitive units of NP provided on the helical nucleocapsids.

Comparisons of primate MxA sequences revealed that L4 is the most divergent part of MxA, whereas the rest of the molecule is highly conserved (20). L4 shows a signature of positive selection during primate MxA evolution, compatible with a role of L4 in recurrent recognition of ancient viruses. A single amino acid (Phe-561) in L4 had a large influence on the antiviral activity of MxA against FLUAV and THOV (20). However, further structural determinants in L4 for viral target recognition remained largely undefined. Here, we demonstrate that at least two motifs in the C-terminal part of L4 are involved. Our data resolve the antiviral interface, allowing MxA to interact with the nucleocapsid component NP. They may be of general relevance for the role of loop L4 in the antiviral action of Mx proteins against a range of pathogenic viruses.

EXPERIMENTAL PROCEDURES

Cells and Viruses—HEK-293T and Vero cells were cultivated in DMEM supplemented with 5% fetal calf serum, 2 mM l-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Thogoto virus (THOV) strain SiAr126 (21), influenza A virus (FLUAV) strain A/Thailand/1/04 (H5N1) (6), and the original strain of La Crosse virus (LACV) (22) were used for the infection experiments.

Plasmids—Human MxA (accession number P20591) or murine Mx1 (accession number P09992) expression plasmids and the respective GTPase-inactive controls (T103A or K49A), as well as MxA-delL4 (lacking amino acids 533–561), were described previously (6, 15). All mutant and chimeric Mx constructs carried a FLAG epitope at the N terminus and were eluted in SDS sample buffer for 5 min at 95 °C and detected by Western blot with specific antibodies for MxA, THOV NP (8), and β-tubulin (Sigma).

Immunofluorescence—To detect LACV nucleoprotein-MxA aggregate formation, Vero cells seeded onto 24-well plates were transfected for 24 h with 250 ng of plasmids expressing FLAG-tagged MxA. The cells were subsequently infected with LACV at an MOI of 10, then fixed with 3% paraformaldehyde, and stained with specific antibodies for MxA, THOV NP (8), and β-tubulin (Sigma).

RESULTS

We recently demonstrated that the loop L4 of human MxA, hsL4, which connects the α3-helix of the stalk with the C-terminal α4-helix, is important for its antiviral activity against influenza viruses. We also showed that L4 is an autonomous module that can transfer the antiviral properties of MxA to the mouse ortholog Mx1 (20). To further characterize the functional requirements of L4 for the antiviral activity of MxA, we generated a chimeric Mx1 construct, Mx1-hsL4, in which the L4 loop of murine Mx1 (mmL4), amino acids 499–547, was replaced by hsL4 of MxA, amino acids 533–578 (Fig. 1A).
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Because of the high variability in the first amino acids of the α4-helix between Mx1 and MxA, we included this region, amino acids 574–578, in our analysis. The chimeric construct, Mx1-hsL4, was first tested for antiviral activity against THOV in a minireplicon assay. FLAG-tagged Mx-encoding plasmids were co-transfected with expression plasmids for the three sub-units of the viral polymerase complex, NP, and an artificial minigenome that encodes firefly luciferase flanked by the non-coding regions of genomic viral RNA segments (20). In contrast to MxA, Mx1 had almost no antiviral effect in this system (Fig. 1B). The chimeric construct, Mx1-hsL4, however, was even more effective against THOV than MxA. Like for wild-type MxA, activity was dependent on GTP binding and hydrolysis, as demonstrated by the lack of antiviral activity of the GTPase-deficient mutant, Mx1-hsL4(K49A) (Fig. 1B). We concluded that this system is ideal to define the critical amino acids in hsL4 for antiviral activity without disturbing the overall architecture of the protein.

Analysis of hsL4 in the Chimeric Mx1-hsL4 Construct—Using Mx1-hsL4, we analyzed N- and C-terminally truncated fragments of hsL4 (Fig. 1A). First, we exchanged only the C-terminal part of Mx1 L4 with the corresponding sequences of MxA-L4, resulting in Mx1-hs(558–578) and Mx1-hs(560–578) as indicated in Fig. 1A. The two constructs containing only the C-terminal half of hsL4 were still active (Fig. 1C). However, loss of position 560 or 561 in hsL4 gradually diminished activity. Furthermore, Mx1-hs(533–571) with a truncation of the distal seven amino acids in hsL4 completely lost activity (Fig. 1C). These results indicate that both the proximal part around position 561 and the distal part of hsL4 that extends into the α4-helix are required for activity. We therefore focused on the C-terminal moiety of hsL4 for further analysis.

Next, we performed an alanine scanning analysis of Mx1-hs(561–578). Two consecutive amino acids in hsL4 were exchanged to alanines or, in the case of pre-existing alanines, to serines, and the resulting hsL4 mutants were tested for antiviral activity in the minireplicon system. Conversion of positions 561/562 and 577/578 to alanines almost completely abolished Mx activity (Fig. 2A). Changing positions 575/576 to alanines reduced the inhibitory effect from ~85% to 50%. Interestingly, mutations in the region in between these two clusters (amino acids 563–574) had no significant effect on the activity of Mx1-hs(561–578) (Fig. 2A).

The most critical positions in hsL4 identified by this analysis were further subjected to single amino acid exchanges to alanine. The results revealed that even single mutations at positions 561 and 562 completely abolished the activity (Fig. 2B). An alanine at position 560 also led to a weak loss of inhibition from ~85% to 60% reduction of polymerase activity. Single alanine exchanges in the distal part moderately weakened the inhibitory effect with the exception of isoleucine to alanine exchange at position 577 that completely abolished the activity of Mx1-hs(561–578) (Fig. 2B). Interestingly, single alanine exchanges of the glutamic acids at position 575 or 576 had no effect, indicating that a single, negatively charged glutamic acid at either position is sufficient. However, introducing aspartic acid for glutamic acid at position 576 in combination with a neighboring alanine at position 575 was deleterious for the antiviral function (Fig. 2B). Thus, the negatively charged residue at this position, as provided by aspartic acid, is not sufficient for anti-THOV activity, indicating a special requirement for glutamic acid that carries a longer side chain.
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We recently demonstrated that at position 561 in hsL4, a bulky aromatic amino acid like phenylalanine or tyrosine is required for antiviral activity (20). Accordingly, we analyzed Ile-577, which is a well-defined position in the MxA structure, localizing in the proximal part of ε-helix, where it points to the hydrophobic core of the stalk (15). It was therefore conceivable that Ile-577 might play a structural role. To evaluate this, Ile-577 was exchanged to other hydrophobic amino acids. Although exchange to alanine abolished activity, exchange to leucine or methionine was well tolerated, suggesting that a hydrophobic amino acid with a longer side chain than alanine was required at this position. In conclusion, our detailed functional analysis of hsL4 corroborated that the proximal positions 561 and 562, as well as the distal region around position 577, were most critical for antiviral activity, whereas the exact sequence of the 12 residues in between these two motifs was apparently not relevant.

To further characterize the role of the central and distal motifs in hsL4 as the major regions conferring the gain of antiviral function of murine Mx1 against THOV, we exchanged only position 526–528 and 544–547 in L4 of Mx1 into the respective residues 560–562 and 575–578 of hsL4 (Fig. 1A, lowest scheme). When compared with wild-type Mx1, the two hsL4 elements in Mx1-hs(560–562 + 575–578) mostly recapitulated the effect of the C-terminal part of hsL4 in Mx1. Mx1-hs(560–562 + 575–578) gained over 75% inhibition of the THOV polymerase activity (Fig. 2C). However, its reduced antiviral activity when compared with Mx1-hs(560–578) might indicate that some minor structural requirements in the linker region between the two critical motifs exist for the functional role of hsL4.

Critical Amino Acid Positions in L4 of MxA—In the next set of experiments, we determined the positions in hsL4 critical for the antiviral activity of full-length human MxA. The positions 560, 561, 562, 576, 577, and 578 were individually mutated to alanines in MxA. In addition, both glutamic acids at positions 575 and 576 were changed to alanines. As an inactive control, we used MxA-dell4 with a deletion in loop L4 from amino acids 533–561 (15). As expected, positions 561, 562, and 577 were most important for antiviral activity in the THOV minireplicon system (Fig. 3A). The same results were obtained in infection experiments in tissue cultures (Fig. 3B). Vero cells were transfected with the MxA expression constructs and then infected with THOV. Viral replication was monitored by the accumulation of NP in MxA-expressing cells, using double immunofluorescence analysis. Activity of MxA was abolished by an alanine exchange at position 561, 562, or 577 (Fig. 3B). Mutation of position 560 to alanine showed only a weak reduction of antiviral activity in the infected cells, although the construct was more or less inactive in the minireplicon system. The activity of mutant MxA with exchanges in position 575, 576, or 578 did not differ significantly from wild-type MxA.

In the next step, we tested the MxA constructs for antiviral activity against FLUAV, using a minireplicon system and infection of cells (Fig. 3, C and D). The outcome of these experiments was almost comparable to that with THOV; especially in the infection experiments (Fig. 3, compare B and D). There were, however, some notable exceptions. First, changing position 560 had no effect on the MxA activity in the infected cells but significantly reduced antiviral activity in the FLUAV minireplicon. Second, mutation of position 562 barely affected antiviral activity against FLUAV, and third, mutations at positions 575 and 576 diminished MxA activity against FLUAV in the minireplicon system and, with a much weaker outcome, in FLUAV-infected cells. The results suggest that specific amino acids in L4 of MxA have important function for the activity against orthomyxoviruses; however, there are slight differences in the positions that dictate antiviral specificity between the two viruses tested.
Role of Loop L4 in the Interaction of MxA with Viral Nucleocapsid Component NP—MxA has been demonstrated to interact with THOV nucleocapsids in co-immunoprecipitation assays (8). Here, the role of L4 for binding to THOV-NP was tested for the most critical positions. 293T cells expressing FLAG-tagged wild-type or mutant MxA were infected with a virus, and the percentage of viral NP-expressing, MxA-positive cells versus the overall amount of MxA-expressing cells was calculated. The percentage of viral NP-expressing, MxA-positive cells versus the overall amount of MxA-expressing cells was calculated by evaluating ~200 cells positive for MxA. The mean values (± S.D.) of three independent experiments, each with two technical replicates, are presented (t test; ***, p < 0.0001; B and D, restriction of THOV and FLUAV replication by MxA in tissue culture. Vero cells were transfected with the indicated MxA expression constructs. At 24 h post-transfection, the cells were infected with THOV (B) or FLUAV, A/Thailand/1/04 (H5N1) (D), at an MOI of 10 for 24 or 5 h, respectively. Then the cells were fixed and stained with specific antibodies for MxA and the viral NPs. The percentage of viral NP-expressing, MxA-positive cells versus the overall amount of MxA-expressing cells was calculated by evaluating ~200 cells positive for MxA. The error bars represent the standard deviation of three independent experiments (t test; ns, not significant; *, p < 0.05; **, p < 0.005; ***, p < 0.0005 in comparison to wild-type MxA).
high dose of THOV. MxA was precipitated from the cell lysates with an anti-FLAG antibody, and co-precipitated viral NP was detected by Western blotting. As expected, NP co-precipitated with wild-type MxA, but not with the MxA-delL4 mutant that lacks a substantial part of L4 and has no antiviral activity (Fig. 4). The interaction of NP with the MxA mutants in L4 correlated with their antiviral activity. Individual exchanges at positions 561, 562, and 577 to alanines abolished co-precipitation of NP, whereas alanine at position 575, 576, or 578 had no effect (Fig. 4). In summary, a few defined positions in L4 are crucial for viral nucleocapsid interaction and antiviral activity, suggesting that the two activities are mechanistically linked.

Interaction of MxA with the Nucleoprotein of La Crosse Virus—MxA inhibits replication of LACV bunyavirus and leads to a redistribution of the viral N protein into perinuclear MxA-N complexes that is a readout for the antiviral effect (14). Here, we used the formation of these perinuclear complexes as a marker to determine the potential of the L4 mutants of MxA to recognize LACV. Cells expressing wild-type MxA, but not with the MxA-delL4 mutant that lacks a substantial part of L4 and has no antiviral activity (Fig. 4). The interaction of NP with the MxA mutants in L4 correlated with their antiviral activity. Individual exchanges at positions 561, 562, and 577 to alanines abolished co-precipitation of NP, whereas alanine at position 575, 576, or 578 had no effect (Fig. 4). In summary, a few defined positions in L4 are crucial for viral nucleocapsid interaction and antiviral activity, suggesting that the two activities are mechanistically linked.

DISCUSSION

Human MxA is an important antiviral effector of the IFN-induced defense against FLUAV and related orthomyxoviruses, such as THOV. In both cases, MxA targets the viral nucleoprotein, most likely in the context of the entire viral nucleocapsid (6–9). We have previously proposed that MxA recognizes surface exposed amino acids in the body domain of FLUAV-NP and defined a surface patch of multiple residues that govern MxA sensitivity or resistance in avian or human FLUAV strains, respectively (7). Although a few amino acid changes in NP appeared sufficient to evade the inhibitory action, the exact nature of the interface in MxA that targets NP remained undefined. Here, we characterized the putative MxA interface and demonstrate that it consists of a central element in the disordered loop L4. L4 protrudes from the compact stalk domain of MxA (16) and is ideally situated to contact cellular or viral substrates. The L4 sequence is surprisingly variable between Mx proteins from different species. Moreover, an analysis of primate MxA proteins revealed that L4 is under strong positive selection, as expected for an interface interacting with fast evolving pathogens (20). Indeed, we could experimentally demonstrate that L4 mediates interactions of MxA with cellular and viral components. Like most large GTPases of the dynamin family, MxA associates with cellular membranes (17, 26), and
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L4 was shown to mediate lipid binding. A stretch of four basic lysine residues in the central part of L4 (Lys-554 to Lys-557) was found to be essential for binding to artificial membranes that contain mostly negatively charged phospholipids (19).

Concerning MxA antiviral function, swapping the L4 sequences between Mx proteins of different species determined the antiviral potency of the resulting chimeric proteins. For example, murine Mx1 in which L4 was replaced by L4 of human MxA (hsL4) gained the antiviral activity typical for wild-type MxA, including co-precipitation of THOV-NP (20), indicating that L4 is an autonomous antiviral module. In the present study, we took advantage of these chimeric Mx constructs because they give a robust gain of function that allowed us to distinguish between bona fide specificity determinants and amino acids that simply disrupt the functional structure when mutated. Using the chimeric Mx1-hsL4 protein, we identified several residues essential for antiviral activity in the C-terminal half of L4, amino acids 560–578, located downstream of the putative membrane interaction site. By mutational analysis, we found a motif around residue 561 and a second motif around position 577, which were both involved in the interaction of MxA with viral NP and for inhibition of viral replication. Interestingly, the 12 residues separating the two motifs could be replaced by the 15 residues of the mmL4 or converted to alanines without major loss of antiviral activity, suggesting only minor structural requirements for this part of L4.

Mutations in the first motif (F561A or G562A) affected the antiviral activity of MxA against orthomyxoviruses. Interestingly, the alanine at position 562 had a deleterious effect on MxA inhibition in THOV- but not in FLUAV-infected cells, indicating a subtle distinction of this element between the two viruses. The shorter glycine residue at position 562 might allow a deeper association of the critical phenylalanine at position 561 with THOV-NP. Both MxA mutants also lost the ability to co-precipitate THOV-NP, demonstrating that NP binding and virus inhibition are closely linked. In contrast, the anti-LACV activity remained intact, as illustrated by the finding that both mutant forms of MxA interacted with LACV-N and formed perinuclear MxA-N complexes in infected cells comparable to wild-type MxA. These results indicate that the first motif in L4 is a specificity determinant for orthomyxoviruses. Furthermore, L4 seems to be highly flexible so that amino acid substitutions in this part of L4 do not lead to a general loss of MxA activity. Residue 561 in L4 has been changed throughout primate MxA evolution and appears to represent a key element in antiviral defense against recurrent viruses (20). Other residues in L4 (Gly-540, Phe-564, Ser-566, and Ser-567) are likewise under positive selection and may serve critical roles against other MxA-sensitive viruses (20, 27).

In contrast to the region containing the first motif, the distal sequences around position 577 show no signature of positive selection but are highly conserved (20). Their deletion or a single amino acid change at position 577 abolished the MxA activity against both orthomyxoviruses and bunaviruses. Ile-577 is located in the beginning of the α4-helix with its residue directed into the hydrophobic core of the stalk (16). It is conceivable that this region is critical for the proper folding of the stalk and for the positioning of L4 to allow an optimal interaction with viral targets.

According to our current model, the disordered L4 acts as a flexible antiviral module in a large molecular machine that is highly conserved. In general, disordered regions in a molecule provide flexibility to contact diverse binding partners. Binding is initially at low affinity, but oligomerization eventually allows multiple interactions that stabilize substrate binding (28, 29). We propose that MxA initially recognizes viral nucleocapsids by binding individual viral nucleoproteins via L4 in infected cells. This then facilitates the formation of multiple MxA-NP contacts, resulting in a stable interaction between MxA oligomers and viral nucleocapsids. MxA-MxA self-assembly is therein of central relevance, and mutations in the intermolecular contact sites that prevent oligomer formation abolish its antiviral function (15, 16). Interactions between MxA oligomers and nucleocapsids might finally result in the mislocalization of the viral components into perinuclear aggregates in LACV-infected cells (14), to a block of nuclear translocation of incoming nucleocapsids of FLUAV and THOV (9, 10), or to disruption of the functional integrity of the nucleocapsids, as suggested for FLUAV (30). It is intriguing to note that a similar mechanism might be at work for the antiviral activity of human MxB against HIV-1 (5). MxB interacts with the capsid protein of HIV-1 and appears to prevent chromosomal integration of the viral DNA (2–4). It remains to be seen whether L4 or some alternative interface in human MxB is involved in the recognition of HIV-1 capsids, as shown here for the antiviral activity of MxA.

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