Effects of allelic variations in the human Myxovirus resistance protein A on its antiviral activity

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ABSTRACT

Only a minority of patients infected with seasonal influenza A viruses exhibits a severe or fatal outcome of infection, but the reasons for this inter-individual variability in influenza susceptibility are unclear. To gain further insights into the molecular mechanisms underlying this variability, we investigated naturally occurring allelic variations of the myxovirus resistance 1 (MXI) gene coding for the influenza restriction factor MxA. The interferon-induced dynamin-like GTPase consists of an N-terminal GTPase domain, a bundle signaling element, and a C-terminal stalk responsible for oligomerization and viral target recognition. We used online databases to search for variations in the MXI gene. Deploying in vitro approaches, we found that non-synonymous variations in the GTPase domain cause the loss of antiviral and enzymatic activities. Furthermore, we showed that these amino acid substitutions disrupt the interface for GTPase domain dimerization required for the stimulation of GTP hydrolysis. Variations in the stalk were neutral or slightly enhanced or abolished MxA antiviral function.
Remarkably, two other stalk variants altered the antiviral specificity of MxA. Variations causing the loss of antiviral activity were found only in heterozygous carriers. Interestingly, the inactive stalk variants blocked the antiviral activity of wt MxA in a dominant-negative way suggesting that heterozygotes are phenotypically MxA-negative. In contrast, the GTPase-deficient variants showed no dominant-negative effect, indicating that heterozygous carriers should remain unaffected. Our results demonstrate that naturally occurring mutations in the human MX1 gene can influence MxA function which may explain individual variations in influenza virus susceptibility in the human population.

Human myxovirus resistance protein A (MxA) is part of the host cell defense against viruses. The innate immune factor shows antiviral activity against a broad range of RNA and DNA viruses including orthomyxo-, bunya-, rhabdo- and paramyxoviruses as well as African swine fever virus and hepatitis B virus (1). Its expression is not directly induced by viruses but by type I (α/β) and III (λ) interferons (1). MxA, a dynamin-like GTPase, can be structurally subdivided into three domains: an N-terminal GTPase domain (G domain), that binds and hydrolyses GTP; an antiparallel four-helical bundle called the stalk; and a connecting three-helical bundle denominated as the bundle signaling element (BSE) (Fig. 1A, B and S1) (2,3).

The stalk and the BSE are responsible for homooligomerization of MxA. The central interface 2 mediates the formation of stalk dimers in a criss-cross pattern. Further assembly of dimers into elongated oligomers is mediated via additional interfaces involving the BSE and the stalk (2,3). Several studies have shown that the antiviral activity of MxA requires an intact BSE and oligomerization (2,3). An unstructured loop (loop L4, amino acids 533–572) connecting the stalk helices α3S and α4S protrudes from the end of the stalk opposite of the G domain (Fig. 1A and B). Residues within this loop L4 were identified as antiviral specificity determinants suggesting that loop L4 acts as an antiviral interface that is involved in viral target recognition (4,5).

In addition, the antiviral function of MxA depends on its GTPase activity (6), with the exception of the antiviral activity against hepatitis B virus (7). Recently, we and others demonstrated that the low basal GTPase activity of MxA is stimulated by GTP-dependent dimerization of the G domains which stabilizes the flexible switch regions to position residues involved in GTP hydrolysis. The interaction between two G domains is mediated via a highly conserved interface, called G interface (8,9). Within an MxA oligomer, formation of G domain dimers is sterically prevented suggesting that such interactions occur between G domains of neighboring oligomers, similarly to dynamin whose G domains form intermolecular interactions across sequential rungs in a dynamin helix (3,10,11). Conformational changes in the G domain induced by GTP hydrolysis were shown to be transmitted to the stalk via the BSE resulting in a power stroke (8,12).

MxA interferes with different steps of viral replication depending on the virus inhibited. The viral target of MxA antiviral activity against orthomyxo- and bunyaviruses is the nucleoprotein, a component of viral nucleocapsids which encapsidates the viral negative strand RNA genome (13,14). In bunyavirus-infected cells, MxA binds and relocates the viral nucleoprotein (N) into perinuclear complexes associated with the smooth endoplasmic reticulum. This sequestration of N is thought to inhibit viral replication (13,15). MxA also interacts with the nucleoprotein (NP) of Thogoto virus (THOV), a tick-borne orthomyxovirus, thereby blocking the nuclear import of THOV nucleocapsids and consequently viral replication (16,17). Similar to THOV, MxA recognizes the NP of influenza A virus (FLUAV) (18,19) and appears to inhibit FLUAV replication by interfering with the transport of incoming nucleocapsids to the nucleus (20,21) and also by affecting later steps of viral replication (19,22). In the case of Vesicular stomatitis virus (VSV) nucleocapsids MxA inhibits viral transcription in the cytoplasm (23,24).

Previously, we demonstrated that naturally occurring allelic variations in the human MX1 gene, which encodes the MxA protein, can negatively affect the antiviral activity of MxA (9). The two examined variations were detected in a small sequencing study of the human MX1 gene (25) and are located in the G interface of MxA. We showed that the variations interfered with G domain dimerization and consequently GTPase
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activity resulting in the reduction or even the complete loss of antiviral activity (9). In the present study, we identified additional, naturally occurring variations using the freely accessible Exome Aggregation Consortium (ExAC) database and determined their impact on the antiviral function of MxA in order to gain a deeper understanding of MxA antiviral action in humans. With MxA being a key restriction factor against FLUAV in humans, as recently demonstrated in a transgenic mouse model of human MxA (26), our data additionally provide insights into potential mechanisms underlying inter-individual variability in susceptibility to severe influenza.

RESULTS

Naturally occurring allelic variations of human MxA—We used the ExAC Browser (http://exac.broadinstitute.org) to detect naturally occurring, allelic variations in the human MX1 gene. This tool searches a publicly available database containing high quality exome DNA sequence data for 60,706 individuals of different ancestries collected from diverse studies and analyzed by the ExAC (27,28). Our analysis revealed 114 synonymous, 269 missense and 14 nonsense (stop codon gained) variants, 4 in-frame deletions, 9 frameshifts and 4 variants located in splice regions (data not shown). Allele frequencies of the variants in MX1 range from 0.00082% (meaning one allele out of 121,412) to 52%. However, most variations that change the amino acid sequence of MxA are extremely rare. About half of the missense variants are singletons (variants seen only once in the data set). Two exceptions are the amino acid exchanges G316R in the G domain and V379I in the stalk of MxA having the highest allele counts of all missense variants (Table 1). Interestingly, most variants are heterozygous. Only for eight missense variants, homozygous carriers can be found in the ExAC database. Again, the two missense variants with the highest number of homozygous carriers are G316R and V379I (Table 1).

In the present study, we focused on missense and nonsense variants and in-frame deletions (Table 1) that are found in distinct populations and are located in structurally interesting sites of the protein. Furthermore, only variants with an allele count of 4 and higher were included in the study in order to select more relevant variants, ideally without excluding those with potential interesting phenotypes due to their rare occurrence. In a recent publication studying the GTPase mechanism of MxA and its role for MxA antiviral function (9), we have already characterized two of these variants located in the G domain, G255E and V268M. They were previously identified by Duc and colleagues (25) studying polymorphisms in the MX1 gene in a small group of 267 healthy individuals (Table 1). These two variants were also found in the ExAC database and were included in the present study for further characterization. In addition, we had a closer look at the variant V470G that was not found in the ExAC database but in the Ensembl (http://www.ensembl.org) and NCBI SNP (https://www.ncbi.nlm.nih.gov/snp) databases (Table 1).

In total, we analyzed 15 variants in this study including the two missense variants with the highest allele counts (Table 1). Four variations are located in the G domain (Fig. 1B and S1) and interestingly, two of them exclusively occur in the Asian population (Table 1). Of the eleven variations located in the stalk (Fig. 1B and S1), one variation lead to a premature stop codon at position 419 and was found only in individuals of African origin with two exceptions (Table 1). We also analyzed one in-frame deletion in the stalk, E516del, and two variants at amino acid positions highly conserved in Mx proteins and dynamin (R408Q and F454C; Fig. S1). Another two variations, F561L and S566Y, are located in the unstructured loop L4 (Fig. 1B and S1).

The wt MxA cDNA we used in previous studies (3,4,9,29) encodes isoleucine at position 379 of the amino acid sequence. In the present study, we therefore analyzed all variations in the MxA(I379) background. Note that the human genome reference (GRCh37/hg19) used by the ExAC encodes valine at this position (see variant V379I in Table 1).

G interface variants affect antiviral activity and GTP hydrolysis—To test the effect of the variations in the G domain on the function of MxA, we initially determined the capacity of these variants to inhibit polymerase activity of a highly pathogenic H5N1 FLUAV (30) using a previously described minirepli-con reporter assay (31). Viral ribonucleoprotein complexes (vRNPs) were reconstituted by co-expressing the viral
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polymerase subunits, viral NP and an artificial RNA minigenome encoding a firefly luciferase reporter gene. These vRNPs are transcribed and replicated by the viral polymerase leading to the expression of firefly luciferase. Co-expression of wt MxA resulted in 85% inhibition of viral polymerase activity (Fig. 2A, white bar). The GTPase-deficient and antivirally inactive mutant T103A (9,32) served as a negative control. The variant G316R with the highest allele count of all missense variations in the G domain was antivirally active comparable to wt MxA, and we therefore did not include it in our further biochemical analyses (Fig. 2A). The variant V268M showed a slightly reduced antiviral activity against FLUAV whereas the variation at position 255 caused a complete loss of the antiviral function of MxA as already demonstrated previously (9). The variant N220D was antivirally inactive as well (Fig. 2A). The observed effects of the G domain variations on the viral polymerase activity in the FLUAV minireplicon reporter assay were confirmed in transiently MxA-expressing A549 cells infected with an MxA-sensitive H7N7 FLUAV encoding a GFP tagged nuclear export protein (NEP) (SC35MNS1_2A_GFP-NEP) (33). MxA positive cells were detected by FACS and the percentage of infected (GFP positive) cells was determined which was greatly diminished in wt MxA-expressing cells (by 93%) compared to cells positive for the inactive control T103A (Fig. 2B, white bar). In this FACS-based assay, we were able to verify the inactive phenotype of the variants N220D and G255E in infected cells.

Interestingly, the variation N220D, along with G255E and V268M, is located in the G interface (Fig. 2C and S1). We and others have shown that this interface mediates GTP-dependent dimerization of the G domains stimulating GTPase activity of MxA (8,9). We tested the influence of the N220D exchange on G domain dimerization and GTPase activity, as we did before with G255E and V268M (9). For the analysis of the variants’ biochemical properties, we used the previously described monomeric stalk mutant M527D (Fig. 1B) (2) to exclude oligomerization via the stalk domains and because it can be purified from bacteria in high quantities without aggregation. M527D cannot assemble into higher order oligomers due to the destruction of stalk interface 2 that is responsible for MxA dimerization (2) (Fig. 2D, M527D Apo). However, M527D still dimerizes via the G domains upon binding of GDP-AlFx, that mimics the transition state of GTP hydrolysis (Fig. 2D), binds guanosine 5’-O-[gamma-thio]triphosphate (GTPγS) with an affinity of about 13 µM (Fig. 2E) and shows high GTPase activity in a protein concentration-dependent manner (Fig. 2F) (2,9). Introduction of N220D into the M527D background disrupted GDP-AlFx dependent G domain dimerization in gel filtration experiments (Fig. 2D). Consequently, nucleotide hydrolysis of M527D/N220D was almost completely abolished (Fig. 2F) correlating with the loss of antiviral activity of N220D (Fig. 2A and B). We obtained the same results for the variant G255E in our previous study whereas V268M showed an intermediate phenotype (9).

Interestingly, all three G interface variations, N220D, G255E and V268M, did not affect GTP binding (Fig. 2E). Thus, the variants in the M527D background were still able to bind GTPγS in isothermal titration calorimetry (ITC) experiments with similar dissociation constants as M527D (Fig. 2E; K_D(M527D) = 13 µM, K_D(M527D/N220D) = 13 µM, K_D(M527D/G255E) = 13 µM, K_D(M527D/V268M) = 17 µM). In conclusion, our data indicate that the loss of antiviral activity caused by the variations N220D and G255E results from a defect in the formation of a G domain dimer which is necessary for the stimulation of GTP hydrolysis.

G interface variants have no dominant-negative effect on the activity of wt MxA—The G interface variants N220D and G255E were exclusively heterozygous in the ExAC database. Only for the third variant in the G interface, V268M, we found one homozygous carrier (Table 1). With N220D and G255E being antivirally inactive, the question arose whether these variations interfered with the antiviral function of the wt allele. To address this question, we initially performed co-immunoprecipitation analyses to test whether the G domain variants were still able to interact with wt MxA. Wt MxA showed a strong self-interaction and the negative control, the oligomerization-deficient mutant M527D, did not interact with wt MxA (Fig. 3A). Wt MxA was also co-precipitated to the same extent by the G interface variants and by the catalytically inactive mutant T103A. Having verified that N220D and G255E interacted with wt
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MxA, we used the FLUAV minireplicon assay to detect potential effects of the variants on the antiviral activity of wt MxA. The G domain variant V268M showing only slight effects on antiviral activity served as an additional control. The variants and control mutants were co-expressed together with wt MxA and antiviral activity was determined. Surprisingly, the antivirally inactive variants N220D and G255E only marginally interfered with the antiviral function of wt MxA, in contrast to the T103A mutant which had a strong dominant-negative effect on wt MxA, as described previously (Fig. 3B) (9). Co-expression of V268M, on the other hand, led to an increase of antiviral activity. No impact on the antiviral activity of wt MxA was observed for the monomeric mutant M527D that was shown to be antivirally inactive (2) (Fig. 3B). These results prompted us to check the effect of the GTPase-deficient variant N220D on the GTPase activity of wt MxA. The amino acid exchange N220D was again combined with the M527D mutation and the resulting MxA mutant expressed in E. coli. M527D at low concentrations was incubated with increasing amounts of M527D/N220D and GTP hydrolysis was monitored. GTPase activity of M527D was stimulated by the GTPase inactive mutant M527D/N220D, as observed previously for the mutant M527D/T103A (9), indicating that formation of the G interface in the heterooligomer is still possible (Fig. 3C).

Variations in the stalk can lead to altered antiviral activity and specificity of MxA—To detect possible consequences of the non-synonymous variations in the stalk for the antiviral function of MxA, we initially tested the antiviral activity of the selected stalk variants against orthomyxoviruses in the minireplicon reporter systems of FLUAV (Fig. 4A) (31) and THOV (SiAr126; Fig. 4B) (5). In order to further analyze the antiviral specificity of the variants, we additionally determined their antiviral activity against VSV. VSV is also a negative single-stranded RNA virus that, however, replicates in the cytoplasm in contrast to orthomyxoviruses. To analyze anti-VSV activity, we performed infection experiments with propagation-incompetent and VSV glycoprotein-pseudotyped VSV-like particles that encode firefly luciferase instead of the viral glycoprotein (G) (VSV* ΔG(Luc)) (34). We measured the luciferase activity in infected cells that directly correlates with viral polymerase activity (Fig. 4D, virus-like particle (VLP) infection). Furthermore, we determined the efficiency of VSV replication and VLP formation by co-transfection of VSV-G together with MxA expression plasmids. Release of infectious VLPs was determined by transferring the supernatants of the co-transfected cells onto naïve indicator cells (Fig. 4E, VLP titration). In addition, we verified our results in a FACS-based assay analyzing MxA-expressing A549 cells infected with FLUAV (SC35MNS1_2A_GFP-NEP) or a GFP-encoding VSV (35) as described for the G domain variants (Fig. 4C and F).

In the assays described above, wt MxA suppressed viral polymerase activity of FLUAV and THOV to 15% and 6%, respectively, compared to the empty vector control (Fig. 4A and B, white bars). Additionally, primary transcription in cells infected with VSV-like particles and VSV-like particle formation were inhibited by 77% and 94% in the presence of wt MxA (Fig. 4D and E, white bars). In cell culture wt MxA restricted viral replication by reducing the percentage of cells infected with FLUAV to 7% and, in the case of VSV-infected cells, to 14% compared to the inactive mutant T103A (Fig. 4C and F, white bars). Among the stalk variants, I379V, Q423K, and Q611H were antivirally active comparable to wt MxA (Fig. 4, light grey bars). Remarkably, four variants (E394K, R408Q, E419ter and F454C) completely lost the ability to inhibit the viral polymerases of both orthomyxoviruses, infection and formation of VSV-like particles and viral replication in cell culture (Fig. 4, red bars). Interestingly, one variation (V470G) even led to enhanced antiviral activity against FLUAV (4.3-fold) and THOV (5.9-fold compared to wt MxA) in the minireplicon systems (Fig. 4A and B, green bars). However, we did not observe this effect in FLUAV-infected cells (Fig. 4C, green bar), likely due to the higher sensitivity of the minireplicon assays. Moreover, the V470G exchange did not affect anti-VSV activity (Fig. 4D-F, green bars).

The two variations F561L and S566Y are located in the loop L4 which is a determinant of MxA antiviral specificity. The amino acid position 561 has already been described to be crucial for the antiviral activity against orthomyxoviruses (4,5). Accordingly, the variant F561L lost its
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Antiviral activity against THOV completely (Fig. 4B, blue bar). The effect of the variation on the inhibition of the FLUAV polymerase was not as pronounced (Fig. 4A, blue bar) and in infected cells only a slight reduction of antiviral activity was observed (Fig. 4C, blue bar). Intriguingly, anti-VSV activity of F561L was not impaired (Fig. 4D, blue bars) confirming the importance of position 561 for MxA antiviral specificity against orthomyxoviruses. In contrast, the second Loop L4 variation in our study, S566Y, had no effect on the antiviral activity of MxA (Fig. 4, blue bars). Strikingly, we identified another variation affecting the antiviral specificity of MxA. In infected cells, the in-frame deletion E516del led to the loss of antiviral activity against VSV (Fig. 4F, yellow bar) whereas it had no impact on the restriction of FLUAV and THOV (Fig. 4A, B and C, yellow bars). In addition, the negative effect of the in-frame deletion on anti-VSV activity of MxA was also detected in the VLP infection experiments, albeit less pronounced. Compared to wt MxA, primary transcription of VSV was 2-fold enhanced and the production of VSV-like particles was 5.5-fold increased in the presence of E516del (Fig. 4D and E, yellow bars).

The GTPase-deficient and antivirally inactive mutant T103A served as a negative control in all assays (Fig. 4, dark grey bars). Taken together, naturally occurring variations in the stalk can affect the antiviral function of MxA and influence its antiviral specificity.

The inactive stalk variants E394K, R408Q and F454C interfere with the antiviral activity of wt MxA in a dominant-negative fashion—The variations in the stalk causing a loss of antiviral activity are located in the first two α-helices and the loop L1S (Fig. 1B and S1). The variations E394K and R408Q are located in the MxA oligomerization interface 3 mediating lateral interactions between two parallel MxA monomers (Fig. S2A and B, grey and yellow monomer) and a third opposing monomer (Fig. S2A and B, blue monomer) (2,3). Interface 3 also involves loop L1S where the variation E394K is located (see yellow monomer). G392 in L1S (yellow monomer) interacts with the loop L2S of a neighboring monomer oriented in parallel (V449 in the grey monomer). The variation R408Q in helix α1C (yellow monomer) is also in the vicinity of loop L2S of the parallel monomer (grey monomer). The variation F454C is located in helix α2S (Fig. S2B, blue monomer). So far, the residue F454 is not known to contribute to one of the known stalk interfaces, although it is highly conserved in Mx proteins and dynamins (Fig. S1) (2,3). The variation at position 419 introducing a premature stop-codon leads to the expression of a non-functional, truncated MxA fragment only consisting of the G domain and a part of the stalk helix α1S (Fig. 1B).

In order to gain insights into why the variants E394K, R408Q and F454C are antivirally inactive, we examined their GTPase activities and their abilities to oligomerize. The variant E394K was insoluble upon expression in E.coli, pointing to a destabilizing effect of the mutation. The variants R408Q and F454C were catalytically active with a slight reduction of their GTPase activities compared to wt MxA (Fig. 5A). In gel filtration experiments, wt MxA eluted as a tetramer, as previously shown by Gao and colleagues (3) (Fig. 5B). R408Q and F454C showed a similar elution profile, indicating that they were still able to form oligomers. The mutant M527D served as a monomeric control (Fig. 5B).

Since only heterozygous carriers of the three variants were found in the ExAC database (Table 1), we studied their potential effect on the activity of wt MxA. In co-immunoprecipitation experiments, the variants E394K and R408Q showed a strong and the variant F454C a slightly reduced interaction with wt MxA compared with the self-interaction of wt MxA (Fig. 5C). The C-terminally truncated variant E419ter did not interact with wt MxA due to the missing stalk. Accordingly, co-expression of E419ter with wt MxA in the FLUAV minireplicon system had no negative effect on the antiviral activity of the wt, comparable to the monomeric control M527D (Fig. 5D). The variants E394K, R408Q and F454C, however, interfered with the antiviral function of wt MxA in a dominant-negative and concentration-dependent fashion like the GTPase-deficient control T103A (Fig. 5D). We also observed a similar dominant-negative effect of these variants on wt MxA in infection experiments with VSV-like particles (data not shown).

Characterization of the MxA stalk variants V470G and E516del—The variation V470G is located in helix α2S of the stalk and close to the interface between the BSE and the stalk of two
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Parallel MxA monomers (Fig. 1B, S2C and D). However, it is unclear how residue 470 could participate in this interface. The in-frame deletion variant E516del has lost the glutamate at position 516 in the stalk helix α3S in close proximity to interface 2 which mediates interactions between two antiparallel MxA monomers (Fig. 1B, S2E and F).

Our preceding experiments investigating the antiviral activity of the stalk variants revealed a role of position 516 in antiviral specificity against VSV (Fig. 4, yellow bars). Therefore, we tested the influence of the variation E516del on the antiviral activity against another negative single-stranded RNA virus replicating in the cytoplasm, Rift valley fever virus (RVFV). Inhibition of RVFV by the variant E516del was significantly reduced compared to wt MxA in infection experiments using VLPs encoding firefly luciferase as a reporter (Fig. 6A). In contrast, the variant V470G did not significantly affect the anti-RVFV activity of MxA which is in accordance with the neutral effect of the variation on the activity against VSV. In order to gain insights into the reasons for the enhanced antiviral effects of the variation V470G on the polymerase activities of FLUAV and THOV, we tested its influence on GTPase activity and oligomerization. This was not possible for the variant E516del because the protein was insoluble upon expression in E. coli. The GTPase activity of the variant V470G was slightly reduced compared to wt MxA (Fig. 6B) and the variant eluted similarly in gel filtration experiments compared to wt MxA (Fig. 6C).

Interestingly, deeper study of the variants V470G and E516del uncovered that both variants showed a reduced capacity to co-precipitate wt MxA in co-immunoprecipitation analyses (Fig. 6D). In order to further examine the interaction pattern of V470G and E516del in living cells, we used a nuclear co-translocation assay (32). Flag-tagged wt MxA showed a cytoplasmic distribution, as previously published (36). However, upon co-expression with an HA-tagged MxA fused to the nuclear localization signal (NLS) of the Simian virus 40 (SV40) large T antigen (HA-NLS-MxA wt), Flag-tagged wt MxA predominantly accumulated in the nucleus colocalizing with HA-NLS-MxA wt (Fig. 6E). In contrast, the self-assembly deficient mutant HA-NLS-MxA M527D did not drag Flag-MxA wt into the nucleus. Analyses of the HA-NLS-MxA variants V470G and E516del with Flag-MxA wt revealed an intermediate phenotype. Although the HA-NLS-MxA variants completely accumulated in the nucleus, co-expressed Flag-MxA wt showed only partial nuclear co-localization. In addition, the self-interaction of the variants V470G and E516del was similarly reduced (Fig. 6E). Thus, the nuclear co-translocation assay confirmed a weakened MxA-MxA interaction of both variants that was already indicated by the loss of wt MxA co-immunoprecipitation (Fig. 6D), suggesting that weakened self-assembly of the MxA variants might influence their antiviral activity.

Additionally, we analyzed the impact of different amino acid exchanges at position 470 on the antiviral action of MxA against FLUAV to gain further insights into the structural requirements at this position causing enhanced antiviral activity. We generated five artificial mutants replacing V470 with non-polar amino acids of increasing size (alanine, leucine and phenylalanine), with the negatively charged amino acid aspartate or with the polar amino acid asparagine. The effects of the mutants on MxA antiviral activity were tested in the minireplicon reporter system of FLUAV (31). The enhancement of antiviral activity induced by glycine at position 470 (Fig. 6F, green bar, 4.9-fold compared to wt MxA) decreased with increasing size of the side chain of the inserted non-polar amino acid. Interestingly, the substitution of the valine with the negatively charged aspartic acid also resulted in enhanced antiviral activity (Fig. 6F, 2.9-fold compared to wt MxA). A similar effect was observed for the mutant V470N with a polar side chain (Fig. 6F, 2.7-fold compared to wt MxA). Remarkably, none of the tested mutations led to the loss or the reduction of wt antiviral activity indicating that position 470 is not crucial for the antiviral action of MxA, but rather plays a modulating role.

The loop L4 variant F561L does not negatively affect the antiviral activity of wt MxA against THOV—The variation F561L caused the complete loss of antiviral activity against THOV (Fig. 4B). Since no homozygous carrier of this variant was identified in the ExAC database (Table 1) and F561L interacted with wt MxA in co-immunoprecipitation analyses (Fig. 7A), we
explored a possible interference of F561L with the antiviral function of co-expressed wt MxA in a THOV minireplicon assay. Surprisingly, co-expression of the variant F561L had only a minor effect on the activity of wt MxA against THOV (Fig. 7B). The antivirally active loop L4 variant, S566Y, served as a control and increased the antiviral activity when co-expressed with wt MxA in the mixing experiment as assumed (Fig. 7B). These data suggest that the presence of inactive F561L molecules in mixed MxA oligomers does not markedly influence the action of wt MxA against THOV.

DISCUSSION

Host genetics plays an important role in inter-individual variability in the course of viral infection (37-39). The aim of the present study was to investigate potential effects of naturally occurring allelic variations in MX1 on the function of the broadly acting antiviral factor MxA, that plays a key role in FLUAV restriction (26). The World Health Organization acknowledged the need for research in this field in its Public Health Research Agenda For Influenza from 2009 by highly recommending to investigate the role of host genetic factors on susceptibility and severity of influenza virus infection (40). Ciancanelli and colleagues described for the first time a genetic cause for life-threatening influenza. A mutation in the interferon regulatory factor 7 (IRF7) resulting in the failure of the transcription factor to upregulate type I and III interferons was detected in a child suffering from severe influenza (41).

Here, we used the publicly available database of the ExAC (27) to search for variations in the human MX1 gene. The 15 variations we selected to study their impact on MxA antiviral action were located in the G domain, the stalk and the unstructured loop L4 covering the structures responsible for GTP hydrolysis, oligomerization and antiviral specificity, respectively (3-5).

We selected four variants located in the G domain (Table 2 for a summary of all data). G316R, the most frequent G domain variant found in the ExAC database, had no effect on the antiviral function of MxA. The variation N220D located in the G interface of MxA, however, caused the loss of antiviral activity against FLUAV. Previously, we and others showed that MxA GTP hydrolysis is stimulated by GTP-dependent dimerization of neighboring G domains forming the G interface (8,9). Biochemical analyses strongly indicate that the amino acid substitution N220D interferes with the formation of a proper G interface leading to defective GTPase activity. These findings are in accordance with the priorly published results for the other inactive G interface variant G255E (9). Notably, although the two G interface variations had an influence on G domain dimerization, GTP binding was not affected supporting previous findings indicating that an intact G interface is not necessary for GTP binding. Residue D253 has recently been shown to stabilize the G interface. Correspondingly, the mutant D253N disrupted the G interface but was still able to bind GTP (8,9). These data strongly corroborate our findings about the GTPase mechanism of MxA and its role for MxA antiviral activity. GTP binding is not sufficient for the antiviral function of MxA. In addition, GTP hydrolysis is needed which is stimulated by the formation of G domain dimers via the G interface resulting in an intramolecular movement of the BSE and the stalk (8,9,12).

The eleven stalk variations analyzed (Table 2 for a summary of all data) had different effects on the antiviral activity of MxA. They were neutral, caused the loss or enhancement of antiviral activity or influenced the antiviral specificity of MxA. The four variations E394K, R408Q, E419ter and F454C led to a complete loss of antiviral activity against RNA viruses replicating in the nucleus (orthomyxoviruses) and cytoplasm (VSV). The variant E419ter was not functional due to the introduction of a premature stop-codon resulting in the truncation of the stalk. An intact stalk, however, is required for MxA oligomerization and viral target recognition, two features necessary for the antiviral function of MxA (2,3,5). At this point, however, we can only speculate why the other variants, E394K, R408Q and F454C, are antivirally inactive. They are still able to oligomerize in gel filtration (R408Q and F454C) and co-immunoprecipitation analyses (E394K, R408Q and F454C). Interestingly, an artificial mutation at position 408 has previously been shown to abolish the antiviral activity of MxA as well, yet by interfering with oligomerization. The mutation R408D disrupted the MxA tetramer and promoted the formation of stable dimers (2). In contrast, R408Q did not affect
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MxA self-assembly indicating that the mere loss of the positive charge at position 408 - the arginine is completely conserved in Mx proteins (Fig. S1) - results in impaired antiviral activity of MxA. The decrease in GTPase activity of the variants R408Q and F454C also cannot account for the complete loss of antiviral function since a comparable reduction in GTP hydrolysis was observed for the antivirally active variant V470G as well, indicating that a certain variability in GTPase activity has no direct influence on the intensity of the antiviral activity. Possibly, unknown features of MxA might be impaired by the variations, like the interaction with a cellular co-factor. Xiao and colleagues showed that interferon treatment modulates the inhibitory effect of MxA on FLUAV replication suggesting the involvement of other ISGs that might act as MxA interacting factors (21). We are currently searching for such MxA-interacting proteins.

One variation in the stalk, V470G, did not affect inhibition of VSV and RVFV, but, intriguingly, displayed increased activity against the viral polymerases of FLUAV and THOV in minireplicon assays. However, this enhanced antiviral effect could not be reproduced in FLUAV-infected cells. Probably, the positive impact of the variation on the inhibition of the FLUAV polymerase is not strong enough to affect restriction of viral replication or to be detected in the FACS-based infection experiments. Some amino acid substitutions at position 470 showed similar effects on the antiviral activity of MxA against FLUAV in minireplicon assays independent of their charge. Notably, all mutants tested were antivirally active. These findings indicate that position 470 might play a modulating but not a central role for the antiviral activity of MxA.

To our surprise, we detected two variations in our study that changed the antiviral specificity of MxA. The variant F561L partly lost its antiviral activity against FLUAV and completely against THOV but was still antivirally active against VSV. F561 located in the loop L4 has previously been reported to be essential for the antiviral activity against orthomyxoviruses (4,5). In an evolution-guided approach Mitchell and colleagues have described the loop L4 as a hotspot for recurrent positive selection in primates indicating a major role of this structure in MxA antiviral specificity (4). In the MxA oligomer all L4 loops stick out on one side forming a surface-exposed interface revealing multiple interaction sites that enable the efficient recognition of viral target structures (2,3). Its structural flexibility and the variability of its amino acid sequence make the loop L4 an ideal structural element that allows a broad antiviral spectrum as well as the evolution of target specificity.

E516del was the second variant displaying altered antiviral specificity. This variant lost the ability to restrict VSV replication in infected cells whereas antiviral action against FLUAV and THOV was not affected. Additionally, inhibition of RVFV by E516del was reduced compared to wt MxA in VLP infection experiments suggesting that the in-frame deletion might impact MxA antiviral activity against viruses replicating in the cytoplasm. Further analyses revealed that the capacity of the variant to self-assemble was considerably reduced. However, it is still unclear how the deletion of one amino acid can alter the antiviral specificity of MxA and how the weakened self-interaction plays a role. The phenotype of the variant may be caused by a locally disturbed stalk structure resulting from the deletion of E516 that may also have an impact on the presentation of specificity-determining residues in the loop L4.

The human MX1 gene is highly conserved which is reflected in the low allele frequencies of the variations that cause changes in the amino acid sequence of MxA (Table 1). The inactive G domain and stalk variants, N220D, G255E, E394K, R408Q, E419ter, F454C and F561L are very rare meaning that only 4 to 33 out of 121,412 alleles in the ExAC database encode the variations. In contrast, the missense variations G316R and V379I with allele frequencies of up to 50% have no effect on the antiviral activity of MxA against orthomyxoviruses and VSV. This shows that deleterious mutations in the MX1 gene are not well tolerated and emphasizes the important role of MxA as an antiviral restriction factor. Recently, Deeg and colleagues showed the key role of human MxA in the host defense against FLUAV in a transgenic mouse model. MxA protected transgenic animals against lethal FLUAV infection whereas Mx negative mice succumbed to the infection (26).
Due to the rare occurrence of the inactive MxA variants, it is not surprising that only heterozygous carriers of the variants are found in the database. Since the formation of oligomers is required for the antiviral function of MxA and the variants were able to form heterooligomers with wt MxA, we were interested in potential dominant-negative effects of the variants on wt MxA. The inactive stalk variants E394K, R408Q and F454C interfered with the antiviral function of wt MxA against FLUAV in a dominant-negative fashion suggesting that active wt MxA may be trapped in non-functional aggregates by the variants. The truncated variant E419ter did not affect wt activity as shown for the monomeric mutant M527D. However, the inactive G domain variants had a surprisingly insignificant effect on the antiviral activity of wt MxA upon co-expression. This indicates that the catalytic centers of N220D and G255E are still intact and, consequently, not only GTPase activity of wt MxA can be stimulated by the variants but also vice versa, allowing a coordinated, GTP hydrolysis-induced powerstroke within the heterooligomer required for antiviral action. Interestingly, the loop L4 variant F561L when co-expressed with wt MxA hardly interfered with the antiviral function against THOV as well. This suggests that a compromised loop L4 (F561L) in some MxA molecules of the heterooligomer does not disturb proper viral target recognition by the wt proportion. Taken together, our experiments indicate that heterozygous carriers of the inactive G domain and loop L4 variants may still benefit from the antiviral effect of one fully functional MXI allele whereas heterozygous carriers of the stalk variants E394K, R408Q and F454C may not be protected despite one intact wt allele.

A study regarding the susceptibility to respiratory syncytial virus (RSV) infection showed that coding variations in the human MXI gene can indeed have an impact on the course of disease. In infants, the MxA variant V379I was associated with a significantly greater risk of developing severe RSV disease (42). Intriguingly, this variation had no effect on the antiviral activity against orthomyxoviruses and VSV in the present study, indicating additional effects of MxA variations on host susceptibility. In future studies, we will investigate whether variations in the human MXI gene causing the loss of MxA antiviral activity are enriched in patients suffering from life-threatening FLUAV infections. Identification of host genes causing inter-individual variability in susceptibility to viral infections will not only contribute to a better understanding of virus host interactions, but will pave the way for new personalized treatment strategies.

**EXPERIMENTAL PROCEDURES**

**Cells, plasmids and antibodies**—HEK-293T, A549 and HeLa cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 µg/ml). For antiviral assays and co-immunoprecipitation analyses, human wt MxA (UniProtKB - P20591, with variation V379I), variants and mutants were fused to a FLAG- or HA-epitope at the N-terminus and cloned into the pCAGGS vector (43) using the restriction enzymes KpnI and XhoI. For the nuclear co-translocation assay, wt MxA and variants of MxA were either Flag-tagged or fused to an HA-epitope followed by the NLS of the SV40 large T antigen at the N-terminus and cloned into the pcDNA3 vector (Invitrogen) using the restriction enzymes KpnI and XhoI or NheI and EcoRI. Variations and point mutations were introduced by PCR using overlapping primers or by using the quick change mutagenesis kit (Stratagene, La Jolla, USA).

The following primary antibodies were used: anti-Flag (Clone M2, mouse monoclonal, F3165, Sigma-Aldrich), anti-HA (rabbit polyclonal, H6908, Sigma-Aldrich), anti-FLUAV NP (Clone AA5H, mouse monoclonal, MCA400, Bio-Rad), anti-THOV NP (rabbit polyclonal, (5)), anti-β-actin (rabbit polyclonal, ab8227, abcam), anti-MxA (mouse monoclonal, M143, (44)). Secondary anti-mouse or -rabbit antibodies used for Western blot analyses were purchased from LI-COR and labeled with IRDye® 800CW or IRDye® 680LT. Anti-rabbit Alexa Fluor® 555 (donkey) and anti-mouse Alexa Fluor® 488 (goat) -conjugated secondary antibodies (A31572 and A11001, Thermo Fisher Scientific) were used for immunofluorescence analyses. Stainings for FACS analyses were performed with an APC-conjugated goat anti-mouse secondary antibody from BD Biosciences (550826).
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Protein expression and purification—Human MxA and the indicated mutants/variants were expressed as N-terminal His6-tagged fusions followed by a PreScission™ cleavage site from a pET28 plasmid in the Escherichia coli BL21 (DE3) Rosetta strain (Novagen) as described in Gao et al., 2010 (2). Bacterial cultures were grown in TB medium at 37°C. At an OD600 > 0.4, cultures were cooled to 18°C and protein expression induced by the addition of 40 µM Isopropyl-β-D-thiogalactopyranosid (IPTG). Following centrifugation, bacterial pellets were resuspended in ice-cold 50 mM HEPES (pH 7.5), 800 mM NaCl, 30 mM imidazole, 5 mM MgCl2, 1 µM DNase I, 2.5 mM β-mercaptoethanol (β-ME), 500 µM Pefabloc SC (Roth) and lysed in a microfluidizer (Microfluidics). A soluble cell extract was prepared by ultracentrifugation at 40,000 g for 45 min at 4°C. After filtration, the supernatant was applied to a Ni-NTA column (GE-Healthcare) equilibrated with 50 mM HEPES (pH 7.5), 400 mM NaCl, 5 mM MgCl2, 45 mM imidazole, 2.5 mM β-ME. The column was extensively washed with 20 mM HEPES (pH 7.5), 800 mM NaCl, 5 mM MgCl2, 45 mM imidazole, 2.5 mM β-ME, 1 mM ATP, 10 mM KCl, and afterwards with 20 mM HEPES (pH 7.5), 400 mM NaCl, 5 mM MgCl2, 45 mM imidazole, 2.5 mM β-ME. Following protein elution by 20 mM HEPES (pH 7.5), 400 mM NaCl, 300 mM imidazole, 5 mM MgCl2, 2.5 mM β-ME, the protein was incubated overnight at 4°C in the presence of 250 µg GST-tagged PreScission protease to cleave the N-terminal His tag. PreScission protease was removed using a GST trap column. The cleaved protein was concentrated and applied to a Superdex 200 16/60 (GE) gelfiltration column in the absence or presence of GDP-AlFx as described in Dick et al. 2015 (9). The running buffer contained 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM MgCl2, but no nucleotides. The MxA mutants and variants were pre-incubated for 15 min with 2 mM GDP-AlFx on ice in gel filtration buffer. 50 µl of a 2 mg/ml protein solution was subsequently applied to the column. A flow rate of 0.5 ml/min was used. Chromatograms were recorded at a wavelength of 280 nm.

Nucleotide-binding studies—Nucleotide dissociation constants were determined at 8°C on a VP-ITC system (MicroCal™, GE Healthcare, Freiburg). 1 mM nucleotide in ITC Buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl2, 5 mM KCl) was titrated in 8 µl steps into a reaction chamber containing 50 µM of MxA(M527D) (or the indicated M527D mutants) in the same buffer, as described in Dick et al. 2015 (9). The resulting heat change was integrated over a time range of 240 sec, and the obtained values were fitted to a standard single-site binding model using Origin®.

Nucleotide hydrolysis assay—GTPase activities of human MxA mutants were determined at 37°C in 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl2, 5 mM KCl, as described in Dick et al. 2015 (9). A saturating concentration of 1 mM GTP was used. Reactions were initiated by the addition of protein to the final reaction solution. For the heteromeric stimulation reactions, the concentration of MxA(M527D) was kept constant at 2.5 µM, and increasing concentrations of the indicated MxA variants were added. At different time points, reaction aliquots were 20-fold diluted in GTPase buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl2, 5 mM KCl) and quickly transferred into liquid nitrogen. Separation of different nucleotides was achieved on a reversed phase high-pressure liquid chromatography (HPLC) system using a Hypersil ODS-2 C18 column. Nucleotide peaks were detected by measuring adsorption at 254 nm and compared with standard nucleotide samples. GTP and GDP in the samples were quantified by integration of the corresponding absorption peaks. Rates derived from a linear fit to the initial rate of the reaction (<40% GTP hydrolysed) were plotted against the protein concentrations and the kobs values were calculated. For data analysis, the program GraFit5 (Erithacus Software) was used.

Analytical gel filtration—The MxA mutants were analysed using a FPLC Akta Purifier (GE Healthcare) equipped with a Superdex 200 10/300 column in the absence or presence of GDP-AlFx as described in Dick et al. 2015 (9). The running buffer contained 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM MgCl2, but no nucleotides. The MxA mutants and variants were pre-incubated for 15 min with 2 mM GDP-AlFx on ice in gel filtration buffer. 50 µl of a 2 mg/ml protein solution was subsequently applied to the column. A flow rate of 0.5 ml/min was used. Chromatograms were recorded at a wavelength of 280 nm.

Minireplicon assay—The polymerase activity of FLUAV, strain A/Vietnam/1203/04 (VN/04; H5N1) (31) was reconstituted in 293T cells seeded into 12-well plates and transfected (JetPEI, Polyplus) with 10 ng pCAGGS expression plasmids for the viral polymerase
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subunits PB2, PB1 and PA as well as 100 ng NP-encoding plasmids. As minigenome, 50 ng of plasmids encoding firefly luciferase in negative sense orientation flanked by 5’- and 3’-UTRs from viral segment 8 (pPolI-FFLuc-RT for FLUAV) were co-transfected. 10 ng pRL-SV40 constitutively expressing Renilla luciferase was added to normalize transfection efficiency. It was previously shown that expression of the firefly luciferase reporter gene correlates with the activity of the reconstituted polymerase complex (45).

For reconstitution of the THOV strain SiAr126 minireplicon system (5), 10 ng pCAGGS expression plasmids for the viral polymerase subunits PB2, PB1 and PA as well as 50 ng NP-encoding plasmids, 50 ng of plasmids encoding firefly luciferase in negative sense orientation flanked by 5’- and 3’-UTRs from viral segment 5 (pHH21-vNP-FFLuc for THOV), and 10 ng pRL-SV40 were co-transfected.

To examine Mx-mediated inhibition of viral polymerase activity, 300 ng (FLUAV minireplicon) or 100 ng (THOV minireplicon) of pCAGGS plasmids encoding N-terminally Flag-tagged MxA mutants or variants were cotransfected. Cells were lysed at 24 h post-transfection and firefly and Renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay (Promega). The experiments were performed three times and each experiment contained technical duplicates. After normalizing firefly to Renilla luciferase activity, one duplicate of the empty vector control was set to 100% and the arithmetic means of the duplicates were calculated. Results are displayed as arithmetic means (± standard deviation) of the means of the duplicates. Western blot analyses were performed to control protein expression using antibodies against the Flag-tag and actin.

Rift valley fever virus (RVFV)-like particle assay—293T cells seeded in 24-well plates were co-transfected with 200 ng of pCAGGS plasmids encoding N-terminally Flag-tagged wt MxA or MxA variants and 100 ng of RVFV-N and –L encoding helper plasmids. 24 h post-transfection cells were infected with RVFV-like particles coding for a firefly luciferase reporter (46). 24 h post-infection cells were lysed and firefly luciferase activity was measured using the Single-Luciferase® Reporter Assay (Promega). The experiment was performed four times and each experiment contained technical duplicates. One duplicate of the empty vector control was set to 100% and the arithmetic means of the duplicates were calculated. Results are displayed as arithmetic means (± standard deviation) of the means of the duplicates. Western blot analyses were performed to control protein expression using antibodies against the Flag-tag and actin. Significance was calculated using one-way ANOVA with Dunnett’s post-hoc test.

FACS analysis of infected, MxA-positive cells—A549 cells seeded in 6-well plates were transfected with 500 ng of pCAGGS expression plasmids encoding N-terminally Flag-tagged wt MxA or MxA variants using Lipofectamine 3000 Reagent (Thermo Fisher Scientific). At 24 h post-transfection cells were infected with VSV*ΔG(Luc) (34) lacking the VSV-G gene and the firefly luciferase reporter plasmid. 24 h post-infection, cells were detached with trypsin and collected using the Single-Luciferase® Reporter Assay (Promega) (VLP infection). Supernatants containing newly produced VLPs due to co-expressed VSV-G were used to infect naïve 293T cells seeded in 12-well plates. 24 h later, firefly luciferase activity in the lysates of the infected 293T cells was determined (VLP titration). The experiment was performed three times and each experiment contained technical duplicates. One duplicate of the empty vector control was set to 100% and the arithmetic means of the duplicates were calculated. Results are displayed as arithmetic means (± standard deviation) of the means of the duplicates. Western blot analyses were performed to control protein expression using antibodies against the Flag-tag and actin.

For reconstitution of the THOV strain SiAr126 minireplicon system (5), 10 ng pCAGGS expression plasmids for the viral polymerase subunits PB2, PB1 and PA as well as 50 ng NP-encoding plasmids, 50 ng of plasmids encoding firefly luciferase in negative sense orientation flanked by 5’- and 3’-UTRs from viral segment 5 (pHH21-vNP-FFLuc for THOV), and 10 ng pRL-SV40 were co-transfected.

To examine Mx-mediated inhibition of viral polymerase activity, 300 ng (FLUAV minireplicon) or 100 ng (THOV minireplicon) of pCAGGS plasmids encoding N-terminally Flag-tagged MxA mutants or variants were cotransfected. Cells were lysed at 24 h post-transfection and firefly and Renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay (Promega). The experiments were performed three times and each experiment contained technical duplicates. After normalizing firefly to Renilla luciferase activity, one duplicate of the empty vector control was set to 100% and the arithmetic means of the duplicates were calculated. Results are displayed as arithmetic means (± standard deviation) of the means of the duplicates. Western blot analyses were performed to control protein expression using antibodies against the Flag-tag and actin.

FACS analysis of infected, MxA-positive cells—A549 cells seeded in 6-well plates were transfected with 500 ng of pCAGGS expression plasmids encoding N-terminally Flag-tagged wt MxA or MxA variants using Lipofectamine 3000 Reagent (Thermo Fisher Scientific). At 24 h post-transfection cells were infected with a GFP-tagged FLUAV reporter virus (SC35MNS1_2A_GFP-NEP) (33) or with a GFP-encoding VSV reporter virus (VSV-GFP) (35) at an MOI of 0.5. The infected cells were detached with trypsin 6.5 h (VSV-GFP) or 10 h (SC35MNS1_2A_GFP-NP) post-infection, washed in 3% FCS/PBS and subsequently fixed as
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well as permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). Intracellular MxA was detected and stained via the anti-MxA antibody M143 and an APC-conjugated secondary antibody. Cells were analyzed on an LSRFortessa™ Cell Analyzer (BD Biosciences) and acquired data were analyzed using FlowJo V10 software (BD Biosciences). After debris and doublet exclusion MxA positive cells were identified and selected and the percentage of virus infected (GFP positive) cells within this population was determined. The GTPase deficient MxA mutant T103A served as an antivirally inactive control. The percentage of T103A and GFP positive cells was set to 100%. Results are presented as arithmetic means (± standard deviation) of three (VSV-GFP) or four (SC35MNS1_2A_GFP-NEP) independent infection experiments.

Co-immunoprecipitation—In order to investigate the interaction of MxA variants with wt MxA, co-immunoprecipitation analyses were performed. 293T cells seeded in 6-well plates were transfected with pCAGGS expression plasmids coding for HA-tagged wt MxA (500 ng) and FLAG-tagged MxA variants/mutants (500 ng) using the JetPEI transfection reagent (Polyplus). 24 h post-transfection, cells were lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 and incubated with anti-Flag-M2 affinity gel (Sigma-Aldrich) for 2 h at 4°C. After extensive washing, the precipitates as well as the whole cell lysates (WCL) were subjected to standard Western blot analysis using antibodies against the Flag-, the HA-tag and actin.

Nuclear co-translocation assay—In order to detect MxA self-interaction, a nuclear co-translocation assay was performed (32). Artificial nuclear forms of wt MxA and variants/mutants carrying an N-terminal HA-tag followed by the NLS of the SV40 large T antigen (HA-NLS-MxA; 300 ng per 24-well) were co-expressed with FLAG-tagged wt MxA or FLAG-tagged MxA variants (100 ng per 24 well) in HeLa cells. At 24 h post-transfection, cells were fixed with 3% paraformaldehyde (15 min, room temperature) and permeabilized with 0.5% Triton X-100 (7 min, room temperature). Flag-MxA and HA-NLS-MxA proteins were stained with antibodies against the Flag- and the HA-tag and Alexa fluor 488 and Alexa fluor 555-conjugated secondary antibodies. Cells were mounted with FluorSave™ Reagent (Merck Millipore) and analyzed at room temperature using a Zeiss Axio Observer.Z1 microscope, a Zeiss objective Plan-Apochromat 40x/0.95 and a Zeiss AxioCam MRC. Images were processed using the software AxioVision (version 4.8.2.0, Zeiss).
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Author contributions: LG, AD, OD and GK conceived this project and planned the experimental design. LG conducted the antiviral and cellular assays. FS assisted LG in performing minireplicon assays and co-immunoprecipitation analyses. AD performed the biochemical characterization of the MxA variants. LG, EB and MM performed database analyses. LG, AD, OD and GK analyzed the data. LG, OD and GK wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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FOOTNOTES
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The abbreviations used are: MX1, myxovirus resistance 1 gene; MxA, myxovirus resistance protein A; G domain, GTPase domain; BSE, bundle signaling element; N, nucleoprotein (from bunyaviruses); NP, nucleoprotein (from orthomyxoviruses); THOV, Thogoto virus; FLUAV, influenza A virus; VSV, Vesicular stomatitis virus; ExAC, Exome Aggregation Consortium; vRNP, viral ribonucleoprotein complex; NEP, nuclear export protein of FLUAV; GTPγS, Guanosine 5′-O-[gamma-thio]triphosphate; ITC, isothermal titration calorimetry; G, glycoprotein; VLP, virus-like particle; RVFV, Rift valley fever virus; NLS, nuclear localization signal; SV40, Simian virus 40; IRF7, interferon regulatory factor 7; SNP, single nucleotide polymorphism; IFITM3, interferon-induced transmembrane protein 3; ISG, interferon-stimulated gene; RSV, respiratory syncytial virus; IPTG, Isopropyl-β-D-thiogalactopyranosid; β-ME, β-mercaptoethanol; HPLC, high-pressure liquid chromatography; MOI, multiplicity of infection; WCL, whole cell lysates; GMPPCP, Guanosine-5′-[(β,γ)-methylene]triphosphate sodium salt.
### TABLE 1

Selected MxA variants in distinct populations.

| Variant | Functional Region | Exome Aggregation Consortium (ExAC)* | Duc et al, 2012 (25) | chr:pos reference/alternate (dbSNP135 rsID) |
|---------|-------------------|--------------------------------------|----------------------|---------------------------------------------|
|         |                   | African allele count out of 10406  | Asian | Latino | European | total allele frequency | number of homozygotes |
|         |                   | 25166 | 11578 | 66740 |         |                  |                     |
| G domain |                   |       |       |       |         |                   |                     |
| N226D   | G interface       | -     | 7     | -     | -       | 0.00005767 | -                   | 21:4261280 A / G    |
| G255E   | G interface       | -     | 5     | -     | -       | 0.00004118 | ✓                   | 21:4261367 G / A     |
|         |                   |       |       |       |         |                   |                     | rs370213700          |
| V268M   | G interface       | -     | 36    | 49    | 9       | 0.0007742   | ✓                   | 21:42613714 G / A    |
|         |                   |       |       |       |         |                   |                     | rs1995457371          |
| G316R   | G domain          | 292   | 6     | 14    | 24      | 0.002784    | -                   | 21:42815731 G / A    |
|         |                   |       |       |       |         |                   |                     | rs52623435            |
| Stalk   |                   |       |       |       |         |                   |                     |
| V379I   | α1N°, IF1         | 4654  | 10999 | 4341  | 38759   | 16893       | 0.5171             | ✓ 21:42817330 G / A  |
|         |                   |       |       |       |         |                   |                     | rs649390              |
| E394K   | L1°, IF3          | -     | 6     | -     | -       | 0.00004944  | -                   | 21:42817975 G / A    |
| R408Q   | α1C°, IF3         | 5     | -     | 1     | -       | 0.00004988  | -                   | 21:42818018 G / A    |
|         |                   |       |       |       |         |                   |                     | rs145288266           |
| E419ter | α1C°, stop gained | 31    | -     | 2     | -       | 0.0002801   | -                   | 21:42818050 G / T    |
|         |                   |       |       |       |         |                   |                     | rs31532725            |
| Q423K   | α1C°              | 8     | -     | -     | -       | 0.0007011   | -                   | 21:42818062 C / A    |
|         |                   |       |       |       |         |                   |                     | rs140505768           |
| F454C   | α2°               | -     | -     | -     | 4       | 0.0003297   | -                   | 21:42821151 T / G    |
| V470G   | α2°, BSE-stalk IF | -     | -     | -     | -       | -           | -                   | 21:42821199 T / G    |
|         |                   |       |       |       |         |                   |                     | rs78721041            |
| E516del | α3°, in-frame deletion | -     | 6     | -     | -       | 0.00004956  | -                   | 21:42824582 GAGA / G |
|         |                   |       |       |       |         |                   |                     | rs748158915           |
| F561L   | L4°               | -     | 1     | -     | 4       | 0.00004119  | -                   | 21:42824721 T / G    |
| S566Y   | L4°               | -     | -     | 10    | -       | 0.00008237  | -                   | 21:42824735 C / A    |
| Q611H   | α4°, IF1          | 61    | -     | 7     | 1       | 0.0005688   | -                   | 21:42830529 G / T    |
|         |                   |       |       |       |         |                   |                     | rs2230454             |

* Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) [May 2015 accessed]
TABLE 2

Summary of the biochemical and antiviral features of the examined variants.

| Variant   | Homozygosity | Interaction with wt MxA | FLUAV   | THOV    | VSV     | RVFV    | Oligomerization | k_{diss} for GTP (min^{-1})a |
|-----------|-------------|------------------------|--------|--------|--------|--------|----------------|-------------------------------|
|           |             |                        | MR     | infection |        |        |                |                               |
| wt        | ✓           | ✓                      | ++     | ++      | ++     | ++     | n.d.           | 21.6 ± 0.2                   |
| N220D     | –           | ✓                      | –      | –       | –      | –      | n.d.           | 13 ± 3                        |
| G255E     | –           | ✓                      | –      | –       | –      | –      | n.d.           | 13 ± 2                        |
| V268M     | ✓           | ✓                      | ++     | ++      | –      | –      | n.d.           | 7.7 ± 0.1                    |
| G316R     | ✓           | ✓                      | ++     | ++      | n.d.   | n.d.   | n.d.           | n.d.                          |

* Experiments were performed with the monomeric M527D mutant.

# Data at 20 μM protein concentration

n.d. indicates not determined.

MR indicates Minireplicon assay.

Infection indicates virus infection experiments analyzed by FACS.

VLP indicates VLP infection assay.

GOMP indicates GDP-AIF2 interaction.
FIGURE LEGENDS

FIGURE 1. **Structure of human MxA and positions of the variations.** (A) Schematic presentation of the MxA primary structure. B, bundle signaling element. (B) Structure of the MxA monomer from the crystal structure (PDB: 3SZR) (3). Domains are color-coded and secondary structure elements are labeled. G domain in orange, BSE in red, stalk in green, unstructured loop L4 visualized as a dotted line in blue. Positions of the G domain and stalk variations are highlighted in blue. Amino acid residues of wt MxA are shown in stick representation. Asterisks (*) mark the positions of previously characterized artificial mutations: T103A in the G domain is GTPase-deficient and M527D in the stalk is a monomeric mutant. Both mutants are antivirally inactive (2,9,32).
Figure 2. Characterization of MxA G domain variants. (A) Antiviral activity of the G domain variants in a FLUAV minireplicon system. 293T cells were co-transfected with expression plasmids for the MxA variants (300 ng) and the minireplicon system of VN/04 including a reporter construct encoding firefly luciferase under the control of the viral promoter. After 24 h, firefly luciferase activity was determined and normalized to the activity of constitutively co-expressed Renilla luciferase. Results are presented relative to the activity in the absence of MxA, the vector control, (see experimental procedures for calculation) and as arithmetic means (± standard deviation) of three independent experiments. Protein expression of FLAG-tagged MxA, viral NP, and actin were determined by Western blot analysis. (B) Restriction of FLUAV replication by G domain variants in tissue culture. A549 cells were transfected with MxA expression plasmids (500 ng) and 24 h later infected with SC35M\textsubscript{NS1\_2A\_GFP\_NEP} (H7N7) at an MOI of 0.5. After fixation of the cells at 10 h post-infection, MxA was stained and cells were analyzed by FACS. MxA positive cells were selected and the percentage of infected (GFP positive) cells was determined. The percentage of GFP positive cells expressing the inactive mutant T103A was set to 100%. Arithmetic means (± standard deviation) of four independent experiments are shown. (C) G domain dimer of GMPPCP-bound (grey) stalkless-MxA (4P4S, G domain A in yellow: residues 70-340, G domain B in blue: residues 68-340) (8). Positions of MxA G domain variations are highlighted in red. Amino acid residues of wt MxA are shown in stick representation. (D) Analytical gel filtration analysis of the indicated mutants in the presence and absence (apo) of GDP-AlFx. (E) Nucleotide binding analysis of monomeric MxA G domain variants by ITC at 8°C. GTP\textsubscript{S} was titrated stepwise into the protein solution. The resulting heat changes were integrated and the obtained values were fitted to a quadratic binding equation (one site binding model). The following \(K_D\) values were derived from the fittings. M527D (black): \(K_D = 13 ± 5\) μM, \(n = 0.83 ± 0.08\); M527D/N220D (red): \(K_D = 13 ± 2\) μM, \(n = 0.50 ± 0.04\); M527D/G255E (blue): \(K_D = 5 ± 2\) μM, \(n = 0.48 ± 0.18\); M527D/V268M (green): \(K_D = 17 ± 3\) μM, \(n = 0.54 ± 0.04\). The MxA constructs showed a varying degree of precipitation in these assays which may explain the reduced binding numbers. (F) Protein concentration-dependent GTPase activities of monomeric M527D (□) and M527D/N220D (○) were determined at 37°C by an HPLC-based assay. The mean \(k_{obs}\) was calculated from two independent experiments for each concentration. The error bars show the range of the two data points.
FIGURE 3. MxA G interface variants have no dominant-negative effect on wt MxA. (A) Co-immunoprecipitation of wt MxA with G domain variants. 293T cells were co-transfected with HA-tagged wt MxA and FLAG-tagged MxA mutants. At 24 h post-transfection, cell lysates were subjected to FLAG-specific immunoprecipitations (IP). Precipitates and whole cell lysates (WCL) were analyzed by Western blot. (B) Effect of MxA G domain variants on the antiviral activity of wt MxA in the FLUAV minireplicon system of VN/04, as described in Fig. 2A. HA-tagged wt MxA (300 ng) was co-transfected with the components of the minireplicon and increasing amounts (50, 100, and 200 ng) of the indicated FLAG-tagged MxA variants. Protein expression was monitored by Western blot analysis. Data are presented as described in Fig. 2A. (C) GTPase activity of M527D can be stimulated by the monomeric G domain mutant N220D. M527D (2.5 µM) was incubated with increasing concentrations of M527D/N220D and GTPase activity was measured as described in Fig. 2F. Vertical lines in the Western blots indicate cuts combining two blots of one experiment run in parallel.
FIGURE 4. Antiviral activity of MxA stalk variants. (A) Antiviral activity against FLUAV in the VN/04 minireplicon system, as described in Fig. 2A. The vector control and the antiviral activity of wt MxA and T103A have already been shown in Fig. 2A. (B) Antiviral activity against THOV in the SiAr126 minireplicon system. MxA variants (100 ng of expression plasmids), the components of the minireplicon system including a reporter construct encoding firefly luciferase under the control of the THOV promoter and Renilla luciferase to monitor transfection efficiency were co-expressed in 293T cells. Firefly luciferase activity was measured in the cell lysates at 24 h post-transfection and normalized to the activity of the Renilla luciferase. Data are presented as described in Fig. 2A. (C) Restriction of FLUAV replication by stalk variants in A549 cells analyzed by FACS, as described in Fig. 2B. The antiviral activity of wt MxA and T103A have already been shown in Fig. 2B. (D-E) Antiviral activity against VSV. (D) 293T cells were co-transfected with FLAG-tagged MxA variants (300 ng) and VSV-G (300 ng). At 24 h post-transfection cells were infected with VSV*ΔG(Luc) at an MOI of 1. Another 24 h later, supernatants were collected, the cells were harvested and firefly luciferase activity was measured (VLP infection). (E) The supernatants containing newly produced VLPs were used to infect naïve 293T cells which were lysed 24 h later to determine firefly luciferase activity (VLP titration). The values are presented relative to the activity in the absence of MxA, the vector control, (see experimental procedures for calculation). Arithmetic means (± standard deviation) of three biological replicates are shown. (F) Restriction of VSV replication by stalk variants in tissue culture. 24 h after transfection with MxA expression plasmids (500 ng), A549 cells were infected with VSV-GFP at an MOI of 0.5 for 6.5 h. Fixed cells stained with an MxA-specific antibody were analyzed by FACS. MxA positive cells were selected and the percentage of infected (GFP positive) cells was determined. The percentage of GFP positive cells expressing the inactive mutant T103A was set to 100%. Results are displayed as arithmetic means (± standard deviation) of three independent experiments. Protein expression of FLAG-tagged MxA, actin, FLUAV and THOV NP was verified by Western blot analyses. Vertical lines in the Western blots indicate cuts combining two blots of one experiment run in parallel. The color code of the bars is explained in the text.
Allelic variations affect MxA antiviral activity

FIGURE 5. Characterization of the MxA stalk variants E394K, R408Q, E419ter and F454C. (A) Protein concentration-dependent GTPase activities of wt MxA (grey), R408Q (red) and F454C (orange) were determined as in Fig. 2F. (B) Analytical gel filtration analysis, as in Fig. 2D, for wt MxA (grey), M527D (black), R408Q (red) and F454C (orange) in the absence of nucleotide. (C) Co-immunoprecipitation of wt MxA with the indicated stalk variants. 293T cells were transfected either with HA-tagged wt MxA (500 ng) or FLAG-tagged MxA variants (500 ng). At 24 h post-transfection, the lysates of cells expressing wt MxA and the indicated mutant were mixed and subjected to FLAG-specific immunoprecipitation (IP). Precipitates and whole cell lysates (WCL) were analyzed by Western blot. * immunoglobulin heavy chain; ** unspecific bands. (D) Effect of the stalk variants on the antiviral activity of wt MxA in the VN/04 minireplicon system, as described in Fig. 2A. HA-tagged wt MxA (300 ng) was co-transfected with the components of the minireplicon and increasing amounts (50, 100, and 200 ng) of the indicated FLAG-tagged variants. Protein expression was monitored by Western blot analysis. Data are presented as described in Fig. 2A. Vertical lines in the Western blots indicate cuts combining two blots of one experiment run in parallel.
Allelic variations affect MxA antiviral activity

FIGURE 6. Characterization of the MxA stalk variants V470G and E516del. (A) Antiviral activity against RVFV. MxA (100 ng) and RVFV-N and -L (50 ng each) were co-expressed in 293T cells. 24 h post-transfection cells were infected with RVF VLPs encoding firefly luciferase as a reporter. Luciferase activities were measured 24 h later and are presented relative to the activity in the absence of MxA, the vector control (see experimental procedures for calculation). Arithmetic means (± standard deviation) of four biological replicates are shown. Western blot analysis was performed to control protein expression of FLAG-tagged MxA and actin. Significance was calculated using one-way ANOVA with Dunnett’s post-hoc test. ns = not significant; **, p ≤ 0.01; ****, p ≤ 0.0001. (B) Protein concentration-dependent GTPase activities of wt MxA (grey; same control as in Fig. 5A) and V470G (green), as in Fig. 5A. (C) Analytical gel filtration of wt MxA, M527D (grey and black; same controls as in Fig. 5B) and V470G (green) in the absence of nucleotide, as described in Fig. 2D. (D) Co-immunoprecipitation of HA-tagged wt MxA (500 ng) with the FLAG-tagged variants (IP) V470G and E516del (500 ng) as described in Fig 5C. Precipitates and whole cell lysates (WCL) were analyzed by Western blot. (E) Nuclear co-translocation of the MxA stalk variants V470G and E516del with wt MxA. Artificial nuclear forms of wt and MxA variants carrying an HA-tag and the NLS of the SV40 large T antigen (HA-NLS-MxA) were co-expressed with FLAG-tagged wt MxA or MxA variants in HeLa cells. At 24 h post-transfection, cells were fixed and stained with antibodies directed against the HA-tag (red) and the FLAG-tag (green). The right column displays the overlay of the two signals. (F) Effect of different amino acid substitutions at position 470 on the antiviral activity of MxA. The antiviral activity of the mutants against FLUAV was determined in the VN/04 minireplicon system, as described in Fig. 2A.
FIGURE 7. The loop L4 variant F561L has no negative effect on wt MxA. (A) Co-immunoprecipitation of HA-tagged wt MxA (500 ng) with the FLAG-tagged variants (IP) F561L and S566Y (500 ng) as described in Fig 5C. Precipitates and whole cell lysates (WCL) were analyzed by Western blot. Vertical lines in the Western blots indicate cuts combining two blots of one experiment run in parallel. (B) Effect of MxA loop L4 variants on the antiviral activity of wt MxA in the THOV minireplicon system, as described in Fig. 4B. HA-tagged wt MxA (50 ng) was co-transfected with the components of the THOV minireplicon system and increasing amounts (25, 50, and 100 ng) of the indicated FLAG-tagged MxA variants. Protein expression was monitored by Western blot analysis.
FIGURE 3

A) Western blot analysis of Flag-MxA, HA-MxA, and their mutants. IP: anti-Flag and WCL (whole cell lysate).

B) FLUAV minireplicon assay (% relative to empty vector).

C) Graph showing the rate constant ($k_{obs}$) for the FLUAV minireplicon assay versus protein concentration (µM).
FIGURE 5

Panel A: Graph showing protein concentration (µM) vs. $k_0$ (min$^{-1}$).

Panel B: Chromatograms for Tetramer and Monomer.

Panel C: Western blot images for IP: anti-Flag and HA-MxA (wt).

Panel D: FluAV minireplicon assay (% relative to empty vector) graph and Western blot images for Flag, HA, NP, and actin.
FIGURE 6

A. RVFV: VLP infection

Firefly luciferase activity (% relative to empty vector)

vector

MxA

T103A

V470G

E516del

MxA

actin

B. kₜₐₜ (min⁻¹) vs. Protein concentration (µM)

wt

V470G

C. Tetramer Monomer

A280 (mAU) vs. Retention volume (mL)

wt

V470G

M527D

D. Flag-MxA

IP: anti-Flag

WCL

Flag

HA

E. Flag

HA

Overlay

vector

Flag-wt

HA-NLS-wt

Flag-wt HA-NLS-V470G

Flag-wt HA-NLS-E516del

Flag-E516del

HA-NLS-E516del

F. FLUAV minireplication assay (% relative to empty vector)

vector

T103A

MxA

V470G

V470L

V470D

V470N

MxA

actin

NP
FIGURE 7

A

B

THOV minireplicon assay (% relative to empty vector)

Flag-MxA

| Flag-MxA | - | T103A | F561L | S566Y | M527D |
|----------|---|-------|-------|-------|-------|
| HA-MxA   | wt| wt    | wt    | wt    | wt    |

Flag

HA

NP

actin
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