Human/Bovine Chimeric MxA-Like GTPases Reveal a Contribution of N-Terminal Domains to the Magnitude of Anti-Influenza A Activity

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Type I interferons (IFN- α/β) provide powerful and universal innate intracellular defense mechanisms against viruses. Among the antiviral effectors induced by IFN- α/β , Mx proteins of some species appear as key components of defense against influenza A viruses. The body of work published to date suggests that to exert anti-influenza activity, an Mx protein should possess a GTP-binding site, structural bases allowing multimerisation, and a specific C-terminal GTPase effector domain (GED). Both the human MxA and bovine Mx1 proteins meet these minimal requirements, but the bovine protein is more active against influenza viruses. Here, we measured the anti-influenza activity exerted by 2 human/bovine chimeric Mx proteins. We show that substituting the bovine GED for the human one in human MxA does not affect the magnitude of anti-influenza activity. Strikingly, however, substituting the human GED for the bovine one in bovine Mx1 yields a chimeric protein with a much higher anti-influenza activity than the human protein. We conclude, in contradiction to the hypothesis currently in vogue in the literature, that the GED is not the sole determinant controlling the magnitude of the anti-influenza activity exercised by an Mx protein that can bind GTP and multimerise. Our results suggest that 1 or several motifs that remain to be discovered, located N-terminally with regard to the GED, may interact with a viral component or a cellular factor so as to alter the viral cycle. Identifying, in the N-terminal portion of bovine Mx1, the motif(s) responsible for its higher anti-influenza activity could contribute to the development of new anti-influenza molecules.

Introduction

IN THE COURSE of his experiments on viral interference, Jean Lindenmann, codiscoverer of interferon (IFN) with Alick Isaacs, discovered by accident an influenza virus resistance trait in A2G mice (Lindenmann 1962). His initial paper prompted a body of studies revealing the existence, in vertebrates in general, of a distinct class of dynamin-like GTPases that are strictly inducible by type I and type III IFNs (Aebi and others 1989; Obar and others 1990; Holzinger and others 2007). Although not all these GTPases appear to possess antiviral activity, they have been collectively termed "Mx proteins" by reference to the acronym forged historically to qualify the resistance phenotype of A2G mice. Mx stands for myxovirus resistance, but in reality, the array of viruses that have proved to be Mx-sensitive extends far beyond ortho- and paramyxoviruses (for a review, see Haller and others 2007), and it is unclear whether the antiviral activity of Mx proteins is an accidental "plus" linked to some undetermined cell function or whether it has evolved to inhibit in each species a set of species-specific pathogens.

The exact mechanism of action of Mx proteins is still a matter of debate. Two strategies have been devised to elucidate them. One strategy has been to detect a physical interaction between an Mx protein and a viral component. This approach has yielded significant advances with regard to the inhibition of the viral cycle of the Thogoto (Kochs and Haller 1999; Weber and others 2000) and La Crosse (Reichelt and others 2004) viruses by the human MxA protein (huMxA). In this precise context, an intimate physical interaction was evidenced between the Mx protein and the viral nucleoprotein (NP), and a causal link was established between this interaction and obstruction of the intracellular traffic of viral components.

The second strategy has been to identify, in Mx proteins, structural motifs that are crucial to their antiviral action. Mx GTPases have a molecular mass of $\sim 75,000$ Da and, similar to dynamins, they display a relatively low affinity for GTP and a high intrinsic rate of GTP hydrolysis. They consist of 3 domains: an N-terminal GTPase domain (G domain, ~ 300 amino acids) that binds and hydrolyses GTP, a middle domain (MD, ~ 150 amino acids) that mediates self-assembly

and oligomerisation, and a C-terminal GTPase effector domain (GED, ~100 amino acids) involved in both self-assembly (Schumacher and Staeheli 1998; Di Paolo and others 1999) and viral target recognition (Zürcher and others 1992; Johannes and others 1997; Lee and Vidal 2002). It is now known that the antiviral activity of an Mx protein requires the integrity of the tripartite GTP-binding site within the G domain (Pitossi and others 1993; Ponten and others 1997), although the ability to hydrolyze bound GTP is dispensable for inhibition of the vesicular stomatitis virus by huMxA (Schwemmle and others 1995), for aggregation of huMxA with the ribonucleoproteins of the Thogoto virus, or for inhibition of this latter virus (Kochs and Haller 1999). On the other hand, recent work has shown that mutations in the MD or GED that prevent huMxA multimerisation abolish the protein's antiviral activity against the influenza A (H5N1) and La Crosse viruses (Gao and others 2010). The ability to bind GTP and the ability to multimerise are, thus, prerequisites for antiviral activity, but these features are clearly not sufficient, as there exist Mx proteins that meet these structural requirements but exert no antiviral activity. On the other hand, the antiviral spectrum of an Mx protein depends on the species from which it is derived. This implies the existence of some other factor. It has proved possible to abolish an Mx protein's antiviral activity or to alter its antiviral spectrum by introducing certain mutations in the GED (for a review, see Lee and Vidal 2002). Briefly, the existing body of published work suggests that to exert antiviral activity, an Mx protein should possess (1) a GTP binding site, (2) structural bases allowing multimerisation, and (3) a specific GED.

The present article describes a functional analysis of 2 chimeric proteins generated from 2 Mx proteins endowed with anti-influenza activity, revealing that motifs remaining to be discovered, located N-terminally with regard to the GED, contribute to controlling the level of antiviral activity.

Materials and Methods

Cells, plasmids, and viruses

Human embryonic kidney cells (HEK293T cells) and African green monkey kidney cells (Vero cells) were grown in an incubator in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS at 37°C under a humidified atmosphere of 5% CO₂–95% air. The pcDNA4-eGFP, pcDNA4-huMxA, and pcDNA4-boMx1 plasmid constructs have been described earlier (Baise and others 2004; Leroy and others 2006). The internal Mx primer sequences used to generate overlapping (~50 nt) 5'- and 3'-terminal fragments of the 2 Mx cDNAs were 5'-ACT GGA AAG CCC CAA AAT-3' (rev, huMxA), 5'-GGA TTG GAA GTA ATG GTT TG-3' (rev, bovine Mx1 [boMx1]), 5'-AGA GAA GGA GCT GGA AGA AG-3' (fwd, huMxA), and 5'-AGA GAA GGA GGC AGA AGA AG-3' (fwd, boMx1), respectively. Combining these internal primers with pcDNA4-specific primers allowed PCR amplification of the 4 expected fragments (respectively 2,094 and 1,971 bp for the huMxA and boMx1 polynucleotides encoding for N-terminal segments; respectively 402 and 356 bp for the huMxA and boMx1 polynucleotides encoding for C-terminal segments). Afterward, the overlap extension PCR method described by Wurch et al. (2000) was used to assemble the coding se-

quences for chimeric Mx proteins and to clone them into the pcDNA4 expression plasmid. All plasmids were sequenced using BigDye chemistry and an ABI 3730 automatic capillary sequencer (Applied Biosystems) according to the manufacturer's instructions. Amino-acid sequences predicted from the coding sequences were identical to those of the boMx1 allelic variant Mx1a (648 residues, accession No. P79135) and canonical huMxA (662 residues, accession No. P20591). All PCR primer sequences and plasmid maps are available on request. The influenza A viruses used were the 2 low-pathogenicity viruses A/swine/Iowa/4/76 (H1N1) and A/chicken/Belgium/150/99 (H5N2). The viruses were propagated in Vero cells to generate the stock solutions.

Generation of Mx-transfected cells

Experiments were carried out in transiently transfected 293T cells. The day before transfection, 293T cells were plated in gelatinized 6-well culture dishes at $3.5\text{--}4 \times 10^5$ cells per well to obtain 50% confluent monolayers. Transient calcium-phosphate-mediated transfection was then carried out by the dropwise incorporation of a mixture containing 5 µg plasmid DNA in 5 µL H₂O, 8 µL CaCl₂ (2 M), 50 µL H₂O, and 63 µL HBS 1× in each well. After overnight incubation, the transfection medium was replaced with fresh medium, and the plates were incubated for another 24 h, after which the virus inoculum was incorporated.

Analysis of mx gene expression

Whole-cell lysates were prepared with Lysis Labelling Binding (LLB) buffer (Eurogentec), proteins were electrophoresed, and immunoblotting was carried out as previously described (Garigliany and others 2009), with polyclonal rabbit antiserum raised against recombinant huMxA (Ronni and others 1993) or recombinant boMx1 (Garigliany and others 2009). For flow cytometry, cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% saponin and 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), blocked with 1% BSA in PBS, and probed with the corresponding primary antibodies followed by Alexa Fluor 647-conjugated anti-rabbit IgG secondary antibody.

In vitro assay of Mx function

To evaluate the anti-influenza activity linked to *mx* gene expression, the percentage of hemagglutinin-positive cells was measured by flow cytometry in infected populations of CaCl₂-transfected 293T cells expressing eGFP, boMx1, huMxA, or a chimeric Mx from the introduced plasmid. Briefly, the transfected cells were infected at a multiplicity of infection of 10. After 1 h of infection, the cells were rinsed with PBS, placed in fresh medium, cultured for another 6 h, and fixed with 4% paraformaldehyde in PBS. To detect the membrane expression of H1 hemagglutinin, an in-house polyclonal antibody raised in rabbits was used as a primary antibody (diluted 1:250 in PBS with 1% BSA). Polyclonal antibodies binding to H1 were then detected with an Alexa Fluor 647-conjugated secondary antibody (diluted 1:500 in PBS/BSA), and the samples were analyzed in an FACS Canto flow cytometer (BD Biosciences). Instrument settings were adjusted with fresh 293T samples in 4 tubes (an unlabelled sample, 2 singly labeled samples, and 1 sample labeled with the mixed antibodies) and FACS Diva™ calculation software (BD Biosciences). The detection and

TABLE 1. MIDDLE DOMAIN/GED JUNCTIONS OF PARENTAL AND CHIMERIC Mx PROTEINS EXAMINED IN THE STUDY

boMx1	515-QMEQLVYCQDQVYRRALQQVREKEAEEEEKNKKSNHYFQSQVSEPSTDEIFQHLLTAYQQE-573
N-bo/hu-C	515-QMEQLVYCQDQVYRRALQQVREKELEEEKKKKSWDFGAFQSSSATDSSMEEIFQHLMAYH-585
N-hu/bo-C	526-QMEQIVYCQDQVYRGALQKVREKEAEEEEKNKKSNHYFQSQVSEPSTDEIFQHLLTAYQQE-573
huMxA	526-QMEQIVYCQDQVYRGALQKVREKELEEEKKKKSWDFGAFQSSSATDSSMEEIFQHLMAYH-585

Amino-acid sequences of human MxA (normal, Swiss-Prot accession P20591), bovine Mx1 (bold, Swiss-Prot accession P79135) and chimeric Mx thereof. The segments presented encompass the junction between middle domain and GED. The size and amino-acid positions of the fragments that were exchanged between the proteins were as follows: 1–538 for N-bo (61.7 kDa) and 539–648 for bo-C (13.3 kDa, numbering according to P79135) and 1–549 for N-hu (62.2 kDa) and 550–662 for hu-C (13.4 kDa, P20591). Sequences were obtained from GenBank™, aligned with ClustalW Version 1.81, and manually adjusted. The N-terminal end of the GED domain is underlined.

GED, GTPase effector domain; huMxA, human MxA protein.

compensation stabilities were tested before each experimental campaign with SetUp™ beads and FACSCanto™ software (BD Biosciences), and dot plots were analyzed with FACS Diva™ (v.5.0.3.; BD Biosciences) software. In a second set of *in vitro* experiments, interference of *mx* genes expression with the biological cycle of influenza A viruses was probed by virus yield reduction assays. To this end, aforescribed transfected 293T cells were infected at a multiplicity of infection of 10⁻². After 1 h of exposure to the inoculum, the cells were rinsed with PBS, placed into fresh medium with trypsin, cultured for another 23 h, and the supernatants were collected and stored at -80°C until use. For determination of viral titers, 10-fold serial dilutions were prepared in Dulbecco's modified Eagle medium, and the viral titers were determined in triplicate by standard plaque assays using Vero cells.

Results

Human-bovine junctional sites of chimeric Mx proteins are available in Table 1 along with size and amino-acid positions of the fragments that were exchanged between the proteins. The success and reproducibility of the calcium-phosphate-mediated transductions were checked by SDS-PAGE analysis of cell lysates and by flow cytometric counting of eGFP- and Mx-positive cells (Fig. 1). To estimate the anti-influenza activity of the chimeric human/bovine Mx proteins, we compared in 293T cells the effects of eGFP (negative control), boMx1, huMxA, N-boMx1/huMxA-C, and N-huMxA/boMx1-C on the proportion of virus-positive cells observed 7 h after infection with a porcine H1N1 and an avian H5N2 strain (Fig. 2). In this experiment, the proportion of infected cells was significantly lower in cells expressing boMx1 (*P*<0.01) or huMxA (*P*<0.05) than in cells expressing eGFP. Replacing the human GED with its bovine counterpart in huMxA did not increase or decrease the anti-influenza activity as compared with huMxA (*P*>0.20). Strikingly, when the human GED was substituted for the bovine GED in boMx1, the resulting chimeric Mx displayed a much higher anti-influenza activity than huMxA (*P*<0.05). We confirmed the result just cited in the case of multicycle infections monitored over a 24-h period (Fig. 3). Expression of either Mx protein caused a significant decrease in the production of progeny virions (*P*<0.05), and this decrease was again more pronounced for boMx1 and for the chimeric Mx made of the bovine N-terminal segment and the human GED (*P*<0.01).

Discussion

The 2 natural Mx proteins whose anti-influenza activity is examined here, huMxA and boMx1, are phylogenetically

very close. When the Mx proteins of vertebrates are classified according to their sequence similarities, these 2 proteins fall into the same subgroup, the so-called “MxA-like” subgroup, along with the ovine, porcine, and canine Mx1 proteins (Haller and others 2010). All of the domains viewed as prerequisites for antiviral activity are present. In huMxA and boMx1, the tripartite GTP binding site (Horisberger and others 1990) is identical (GDQSSGKS, DIPG, and TKPD), and the 3 interfaces involved in multimerisation show high similarity (Gao and others 2010). With one exception (D377E), interface No. 1 of boMx1 consists of the same amino acids as those of huMxA. Only 3 of the 9 residues involved in interface No. 2 (L520S, I530L, and Q606R) and 4 of the 10 residues involved in interface No. 3 (T395F, E398Q, K435Q, and R452K) are different in boMx1. These minor differences do not prevent the bovine protein from multimerising, as shown by the systematic detection of aggregates by immunofluorescence (Baise and others 2004). On the other hand, neither of these proteins has a nuclear localization signal and accordingly, both of them accumulate in the cytoplasm. Despite these structural convergences, the 2 proteins have very different antiviral spectra. For example, huMxA inhibits

FIG. 1. Expression of *mx* genes after calcium-phosphate-mediated transfection of HEK293T cells. Cells were transfected for 24 h with a plasmid bearing the indicated gene. The expected Mx proteins were sought by immunoblotting of cell lysates and by flow cytometric counting of positive cells. The reported values are means ± SD of at least 3 distinct experimental campaigns. Cells and cell lysates were prepared as described under the Materials and Methods section. A single band was retrieved from each blot, each with the expected size. In each experimental campaign, >95% of the treated cells were successfully transfected, and the transfection efficiency was similar for the 5 plasmids, as determined by regular one-way ANOVA, with *P*>0.05.

FIG. 2. Monitoring of the anti-influenza activity exerted by 2 natural Mx proteins and 2 reciprocal chimeras of these proteins *in vitro*. Percentage of hemagglutinin-positive cells 7 h after exposure of 5 distinct calcium-phosphate-transfected 293T cell monolayers to influenza A virus subtypes H1N1 and H5N2. Expression of the indicated exogenous protein was driven by the corresponding plasmid introduced 48 h before infection, each transfection resulting in >95% expressing cells (Fig. 1). Cells fixed with 4% paraformaldehyde were then analyzed with the BD FACSCanto flow cytometer after immunostaining with in-house anti-H1 rabbit antiserum followed by Alexa Fluor 647-conjugated goat anti-rabbit IgG. The reported values are means $\pm$ SD of at least 4 distinct experimental campaigns. The indicated significant differences were determined by regular one-way ANOVA followed by the Bonferroni post-test, with * P < 0.05 and ** P < 0.01 and NS, P > 0.20.

the cycles of many viruses of the Paramyxoviridae family (Haller and Kochs 2011), whereas boMx1 has no effect at all on them (Leroy and others 2007). On the other hand, although both Mx proteins have the ability to hinder the biological cycle of influenza viruses, huMxA exerts a dramatically lesser activity against them than boMx1 (Garigliany and others 2008). This result is confirmed here (Figs. 2 and 3). Until now, such differences in the antiviral spectrum and in the level of antiviral activity have been attributed to the GED (for a review, see Lee and Vidal 2002).

Here, we have tested this hypothesis in the case of anti-influenza activity. Replacement of the GED of huMxA with that of boMx1 neither increased nor decreased hindrance of the viral cycle (Figs. 2 and 3). This result suggests that the human and bovine GEDs are functionally comparable as regards their ability to counter influenza viruses. In other words, the bovine GED does not seem to provide a material basis for the greater anti-influenza activity and narrower antiviral spectrum of boMx1 nor does the GED of the human protein seem to be responsible for its lesser anti-influenza

FIG. 3. Influenza A virus subtypes H1N1 and H5N2 are repressed in HEK293T cells transiently expressing 2 natural Mx proteins and 2 reciprocal chimeras of these proteins *in vitro*, as judged from the virus titers retrieved in virus yield reduction assays from supernatants sampled 24 h pi. Titers were calculated by the Reed-Muench method. The reported values are means $\pm$ SD of 2 distinct experimental campaigns. The indicated significant differences were determined by regular one-way ANOVA followed by the Bonferroni post-test, with * P < 0.05 and ** P < 0.01 and *** P < 0.001 and NS, P > 0.20.

activity. This view is supported by the dramatically increased anti-influenza activity of the reciprocal chimera, that is, boMx1 equipped with the human GED instead of the bovine GED. On the basis of these results, we conclude that the anti-influenza activity of an Mx protein which can bind GTP and multimerise is not determined exclusively by its GED. This suggests that 1 or several motifs within the G or MD of boMx1 may interact with a viral component or with a cellular factor so as to alter the biological cycle of the virus.

The influenza A virus NP was recently shown to be the target of Mx action, and the possibility of a direct physical interaction between huMxA and NP was previously demonstrated in immunoprecipitation assays under cross-linking conditions (Turan and others 2004; Zimmermann and others 2011). Furthermore, the structural details of MxA oligomerization have recently been elucidated (Gao and others 2010), and the functional model that was inferred predicts that MxA tetramers oligomerize on the surface of viral nucleocapsids, thereby inhibiting the transcriptional activity of the viral polymerase complex (Haller and others 2010; Gao and others 2011). Thus, small changes in the amino-acid composition of NP are supposed to affect the strength of MxA binding, its oligomerization, and its antiviral activity. The present findings are compatible with a complementary version of the same model: subtle changes in the N-terminal segment of an Mx protein may affect NP/Mx interaction and therefore, affect antiviral activity. Despite repeated attempts, however, we were never able to highlight the existence of a physical interaction between huMxA or boMx1 and NP, which suggests that the interaction might also be indirect, involving a cellular factor. Identifying in the N-terminal portion of bovine Mx1 the motif(s) responsible for its higher anti-influenza activity could contribute to the identification of this putative cell partner, which, in turn, could lead to the development of new anti-influenza molecules.

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Author Disclosure Statement

No competing financial interests exist.

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