

## Short Communication

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# Human MxA protein confers resistance to double-stranded RNA viruses of two virus families

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The interferon-induced human MxA protein belongs to the dynamin superfamily of large GTPases and accumulates in the cytoplasm. MxA is a key component of the innate antiviral response and has previously been shown to inhibit several viruses with single-stranded RNA genomes of both polarities and a DNA virus. In addition, MxA also targets two double-stranded RNA viruses, *Infectious bursal disease virus* and a mammalian reovirus as shown in this study. Thus, the antiviral spectrum of human MxA is broader than hitherto suspected. Interestingly, virus growth was not affected in cells expressing MxA(E645R), a mutant form of MxA that showed antiviral activity against orthomyxoviruses.

The human MxA protein is an interferon (IFN)-induced 76 kDa protein which harbours GTPase activity (Horisberger, 1992). The antiviral activity of MxA was shown first for *Influenza A virus* and vesicular stomatitis virus (VSV; Pavlovic *et al.*, 1990). MxA accumulated in the cytoplasm of IFN-activated cells and showed antiviral activity against members of several virus families with negative-stranded RNA genomes (Lee & Vidal, 2002), positive-stranded RNA genomes (Chieux *et al.*, 2001) and a virus with a DNA genome (Gordien *et al.*, 2001). The mechanism by which human MxA protein is able to inhibit virus replication is not fully understood. It has experimentally been shown that MxA targeted the viral nucleocapsids of *Thogoto virus* (Kochs & Haller, 1999a, b) and La Crosse virus (Weber *et al.*, 2000; Kochs *et al.*, 2002; Reichelt *et al.*, 2004) or interfered with amplification of viral RNA of *Semliki Forest virus* (Landis *et al.*, 1998). In addition, in its purified form, MxA was able to inhibit transcription of VSV RNA (Schwemmler *et al.*, 1995). Furthermore, the inhibitory effect of MxA seemed to be dependent on the cellular model used, as described for *Measles virus* (Schnorr *et al.*, 1993; Schneider-Schaulies *et al.*, 1994). This indicated that suppression of viral infection by human MxA did not occur by one single mechanism.

In order to assay whether MxA is able to influence replication of double-stranded RNA (dsRNA) viruses, two viral systems were investigated, the *Infectious bursal disease virus* (IBDV) and a human reovirus. IBDV is member of the family *Birnaviridae* (Delmas *et al.*, 2004) and the cause of a worldwide economically important immunosuppressive disease in chicken livestock. The genome of birnaviruses consists of two segments, A and B, which are localized

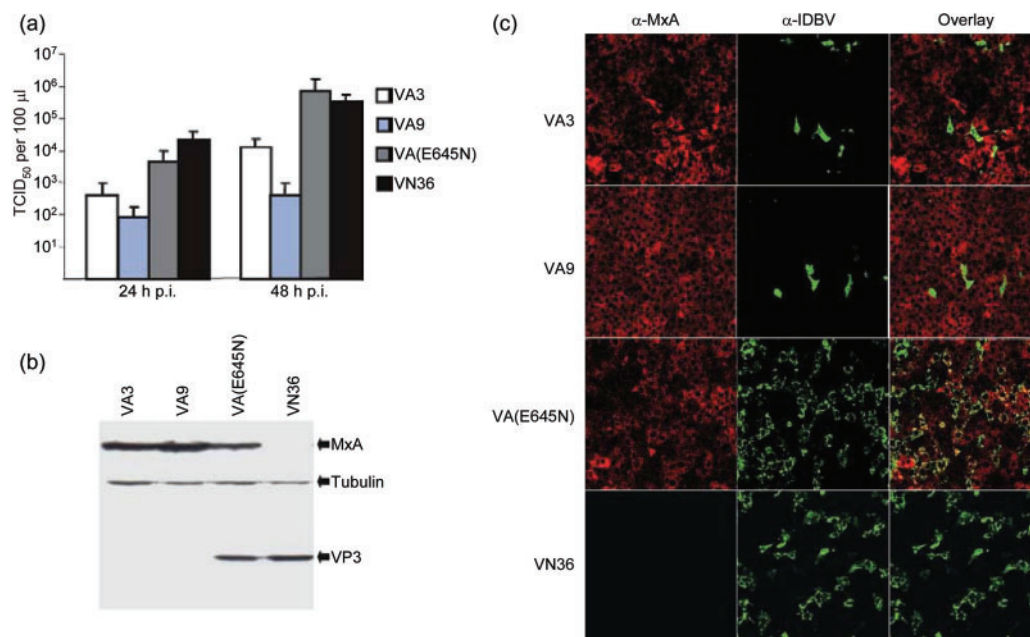
within a single-shelled icosahedral capsid of 60 nm diameter. Few reports have been published regarding Mx protein of poultry. For duck Mx protein, no enhanced antiviral activity was observed after influenza virus infection (Bazzigher *et al.*, 1993). This is an interesting finding since Ko *et al.* (2002) described that cloned chicken Mx showed antiviral activity against influenza virus and VSV. However, the antiviral effect was dependent on cells obtained from different chicken breeds. In subsequent investigations, they showed that aa 631, located at the carboxy terminus of the protein, corresponded with the positive antiviral phenotype against VSV (Ko *et al.*, 2004). In fish, it was shown that expression of Mx1 protein of Atlantic salmon inhibited the replication of *Infectious pancreatic necrosis virus*, another member of the family *Birnaviridae* (Larsen *et al.*, 2004).

The second dsRNA virus used was the mammalian reovirus which belongs to genus *Orthoreovirus*, one of the six genera of the family *Reoviridae*. Mammalian reoviruses are non-enveloped viruses that replicate in the cytoplasm of infected cells and contain 10 dsRNA genome segments surrounded by two protein shells termed the outer capsid and the core of 70–80 nm in diameter (Tyler, 2001). It is known that two IFN-induced proteins contribute to the innate immune response due to reovirus-induced host cell shutoff, the dsRNA-activated protein kinase (PKR) and RNase L (Smith *et al.*, 2005). This study shows that these cellular factors are not the sole determinants of the cellular innate antiviral activity against reovirus and it contrasts with reports showing well-documented antiviral activities of PKR and RNase L (De Benedetti *et al.*, 1985; Li *et al.*, 1998; Balachandran *et al.*, 2000). Smith *et al.* (2005) assumed an additional cellular factor responsible for the antiviral activity against mammalian reovirus. Studies with reovirus including MxA have so far not been described.

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In order to assay whether human MxA has antiviral activity against dsRNA viruses, recombinant African green monkey kidney (Vero) cells were used, which constitutively express wild-type MxA (clones VA9, VA3) or the mutant MxA(E645R) [VA(E645R)], and control cells without MxA (VN36) which contain only the cloning vector (Frese *et al.*, 1996). Cells were incubated with Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum and 2 mg G418 ml<sup>-1</sup> (Invitrogen). Cells were seeded into 24-well plates, grown to confluence and infected at an m.o.i. of 1 with the IBDV strain D78 (Mundt, 1999). After incubation for 60 min at 37 °C supernatants were removed, cells were rinsed once with serum-free medium and 1 ml G418-containing medium was added. At 24 and 48 h supernatants were removed and stored at -70 °C. The TCID<sub>50</sub> was measured on chicken embryonic cells (CEC) as described before (Mundt *et al.*, 2003). As shown in Fig. 1(a), virus yield in the supernatant was reduced about 100- (VA3) to 500-fold (VA9) in comparison to cells expressing the mutated MxA(E645R) [VA(E645R)] or the control cells (VN36). These results indicated that human

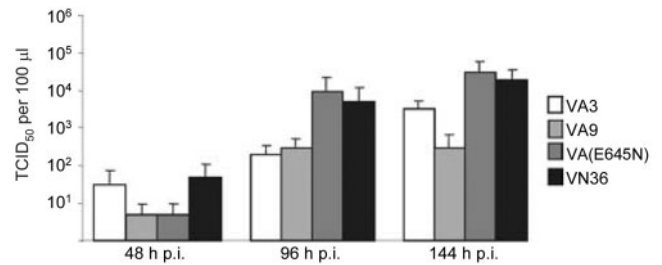
MxA protein had a strong antiviral effect on IBDV replication. In order to exclude that this finding was due to different amounts of MxA proteins, Western blot analysis was performed using lysates of simultaneously infected cells at 48 h post-infection (p.i.; Fig. 1b). After separation by SDS-PAGE (10% gel), proteins were blotted on nitrocellulose membranes, blocked with 5% skim milk solution in TBST [10 mM Tris/HCL, 150 mM NaCl (pH 8.0)] containing 0.05% (v/v) Tween 20 and simultaneously incubated with mAb raised against VP3 of IBDV (Granzow *et al.*, 1997), MxA (Flohr *et al.*, 1999) and cellular  $\alpha$ -tubulin (Sigma). After incubation with anti-mouse horseradish peroxidase-conjugated goat antibodies (Sigma) and application of SuperSignal West Pico chemiluminescent substrate (Perbio Science), it was observed that the amount of MxA protein was very similar in lysates of the different cell lines. VP3 of IBDV was detectable only in lysates of those cells where either the mutated MxA(E645R) or no human MxA protein was expressed (VN36). That equal amounts of proteins were analysed was proven by the presence of similar amounts of  $\alpha$ -tubulin.



**Fig. 1.** Antiviral activity against IBDV in MxA-expressing African green monkey kidney (Vero) cells (VA3, VA9), cells expressing a mutant of MxA [VA(E645R)] and control cells without MxA (VN36). (a) Cells were infected as described in the text. Supernatants removed at the indicated times p.i. (h p.i.) were used for determination of the viral titre indicated in TCID<sub>50</sub> per 100 µl on CEC as described earlier (Kaerber, 1931). Values were plotted exponentially on the vertical axis. Mean titres and sd (error bars) from three independent experiments are indicated. (b) For Western blot analysis, cellular lysates of the Vero cell lines infected with IBDV strain D78 were tested for the presence of IBDV protein VP3, MxA and  $\alpha$ -tubulin by simultaneous incubation with three mAb (anti-VP3; anti- $\alpha$ -tubulin; anti-MxA) after separation by SDS-PAGE (10% gel). The binding of the antibodies was detected by application of an anti-mouse peroxidase-conjugated antibody followed by chemiluminescence assay. The positions of the appropriate proteins are marked by arrows. (c) Vero cell lines were simultaneously infected with IBDV strain D78. At 48 h p.i., cells were fixed with ice-cold ethanol and processed for immunofluorescence using a mouse anti-MxA mAb and a polyclonal rabbit anti-IBDV serum. The binding of the antibodies to the appropriate antigens was visualized by using anti-mouse DTAF-conjugated antibody and swine anti-rabbit Cy3-conjugated antibody. Microscopic examination was performed using a confocal laser scanning microscope LSM 510 (Zeiss).

The simultaneous incubation with three different antibodies was possible due to the different molecular masses of the proteins analysed. In order to analyse to what extent the replication was inhibited, recombinant cell lines were simultaneously infected and immunofluorescence analysis was performed (Fig. 1c) at 48 h p.i. by confocal laser scanning microscopy using an LSM 510 (Zeiss). To this end, cells were fixed with ice-cold ethanol for 10 min, rinsed with PBS and incubated with a polyclonal anti-IBDV serum obtained from a rabbit (Granzow *et al.*, 1997) and the anti-MxA mAb, followed by incubation with anti-mouse DTAF-conjugated swine antibodies and anti-rabbit Cy3-conjugated goat antibodies (Dianova). Specific fluorescence was observed in a very low number of VA3 or VA9 cells infected with IBDV, whereas in the other cell lines used [VN36, VA(E645R)] a large number of cells showed IBDV antigen. This finding confirmed the results from Western blot analysis where VP3 was not detectable in lysates of infected VA3 and VA9 cells, probably due to the low amount of viral protein present. The observation that in principle the MxA-expressing cells are permissive for IBDV infection and that the virus was amplified in the cells, although to a lower number as shown by the growth kinetics, raised the question about the mechanism of the antiviral effect. In order to assay whether there is a protein-protein interaction as described for *Thogoto virus* (Kochs & Haller, 1999a), immunoprecipitation assays were performed. IBDV and MxA proteins couldn't be co-precipitated with appropriate antibodies, whether GTP $\gamma$ S was present or not (data not shown). In addition, during immunofluorescence studies using IBDV protein-specific mAbs raised against VP2, VP3, VP4 (Granzow *et al.*, 1997) and VP5 (Mundt *et al.*, 1997) and a polyclonal anti-MxA serum from a rabbit (Ponten *et al.*, 1997), no co-localization was observed by confocal laser scanning microscopy. No co-localization was observed by immunofluorescence using anti-VP1 antiserum (Birghan *et al.*, 2000) and the anti-MxA mAb either. In conclusion, the mechanism by which MxA inhibited IBDV replication remains unknown.

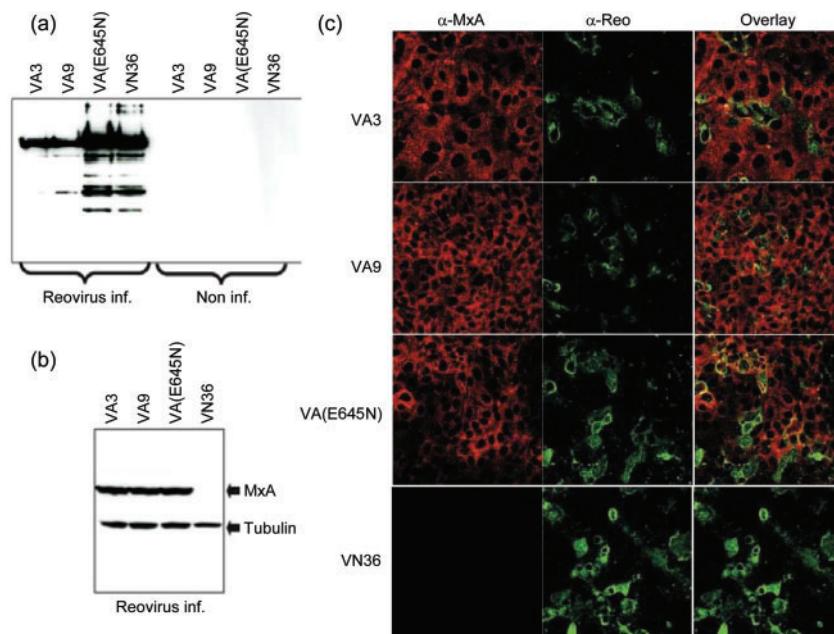
To analyse whether this phenomenon of interference of MxA expression with virus replication was IBDV-specific, replication studies with a mammalian dsRNA virus were performed. The mammalian orthoreovirus type 3 strain Dearing (T3D, a generous gift of Terence Dermody, Vanderbilt University, USA) was passaged once on Vero cells. The four different cell lines [VA9, VA3, VA(E645R) and VN36] were cultivated as described above. Confluent cells were simultaneously infected at an m.o.i. of 1 with the T3D strain for analysis using three methods (Western blot, immunofluorescence and virus growth kinetics). The viral titres were analysed at different time points after infection (48, 96, and 144 h p.i.) by determination of the TCID<sub>50</sub> using unmodified Vero cells (VERO76, Collection of Cell Lines in Veterinary Medicine, RIE 228) cultivated in DMEM containing 10% fetal calf serum (Fig. 2). No significant differences in virus titres were detected at 48 h



**Fig. 2.** Replication of the human reovirus strain T3D in African green monkey kidney (Vero) cells (VA9, VA3), cells expressing a mutant of MxA [VA(E645R)] and control cells without MxA (VN36). Cells were infected with an m.o.i. of 1. The supernatants were harvested at the time points indicated (h p.i.) and the TCID<sub>50</sub> was determined by titration on non-modified Vero cells as described earlier (Kaerber, 1931). Values were plotted exponentially on the vertical axis. Mean titres are shown and SD (error bars) from three independent experiments are indicated.

p.i. At later time points, viral multiplication was reduced 50-fold (VA3, VA9) at 96 h p.i. and 10- (VA3) to 100-fold (VA9) at 144 h p.i. in supernatants of infected wild-type MxA-expressing cells in comparison to cells expressing either the mutant protein [VA(E645R)] or cells lacking MxA (VN36). Western blot analysis of cellular lysates at 96 h p.i. using a rabbit polyclonal anti-reovirus serum (a generous gift of Terence Dermody, Vanderbilt University, USA) and anti-rabbit horseradish peroxidase-conjugated goat antibodies substantiated this finding, since the amount of viral proteins was reduced in cells expressing the wild-type MxA protein (Fig. 3a). In addition, these data were confirmed after analysis of infected cells by immunofluorescence (Fig. 3c), since the number of infected cells was reduced in MxA-expressing cell cultures. This showed that the presence of human MxA protein was also able to reduce replication of human orthoreovirus type 3 strain Dearing. Whether MxA could be one of the additional cellular factors assumed to be responsible for the antiviral activity against mammalian reovirus (Smith *et al.*, 2005) needs to be further elucidated. Reovirus was a strong inducer of IFN expression (Tytell *et al.*, 1967), but the results obtained were very likely not the result of IFN-induced genes since Vero cells contain a genetic defect for expression of IFN (Emeny & Morgan, 1979). The effect observed was probably due to the action of human MxA on reovirus replication by an unknown mechanism.

Here I describe for the first time that human MxA is able to interfere with replication of dsRNA viruses. This finding added dsRNA viruses onto the list of viruses inhibited by the human MxA protein. Interestingly, the mutant MxA(E645R) showed no inhibitory effect on replication of the dsRNA viruses investigated, as has been shown earlier for other viruses. In those cases where an inhibitory effect of MxA(E645R) was observed, the viruses replicated in the cellular nucleus (Zürcher *et al.*, 1992; Frese *et al.*, 1995).



**Fig. 3.** Presence of reovirus antigen in recombinant Vero cells. Recombinant Vero cell lines [VA9, VA3, VA(E645R) and VN36] were infected (reovirus inf.) with the human reovirus strain T3D strain or left mock-infected (non inf.). Equal amounts of cellular lysates obtained at 96 h p.i. were separated by SDS-PAGE (10% gel) and incubated with rabbit anti-reovirus serum (a) or two mAb (anti-MxA and anti- $\alpha$ -Tubulin) (b). The binding of the appropriate antibodies was detected by incubation with species-specific horseradish peroxidase-conjugated antibodies followed by enhanced chemiluminescence assay. (c) Recombinant Vero cell lines were infected and processed for immunofluorescence. Fixed cells were incubated with rabbit anti-reovirus serum ( $\alpha$ -Reo) and an anti-MxA mAb ( $\alpha$ -MxA). The antibodies bound were detected by incubation with species-specific conjugated antibodies (DTAF, rabbit; Cy3, mouse) and visualized with a confocal laser scanning microscope.

Therefore, the GTPase activity which is retained in the mutant cannot be the sole determinant for the inhibitory effect.

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