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Mx Is Dispensable for Interferon-Mediated Resistance of Chicken Cells against Influenza A Virus▽

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The type I interferon (IFN) system plays an important role in antiviral defense against influenza A viruses (FLUAV), which are natural chicken pathogens. Studies of mice identified the Mx1 protein as a key effector molecule of the IFN-induced antiviral state against FLUAV. Chicken *Mx* genes are highly polymorphic, and recent studies suggested that an Asn/Ser polymorphism at amino acid position 631 determines the antiviral activity of the chicken Mx protein. By employing chicken embryo fibroblasts with defined Mx-631 polymorphisms and retroviral vectors for the expression of Mx isoforms in chicken cells and embryonated eggs, we show here that neither the 631Asn nor the 631Ser variant of chicken Mx was able to confer antiviral protection against several lowly and highly pathogenic FLUAV strains. Using a short interfering RNA (siRNA)-mediated knockdown approach, we noted that the antiviral effect of type I IFN in chicken cells was not dependent on Mx, suggesting that some other IFN-induced factors must contribute to the inhibition of FLUAV in chicken cells. Finally, we found that both isoforms of chicken Mx protein appear to lack GTPase activity, which might explain the observed lack of antiviral activity.

The chicken is a natural host for influenza A virus (FLUAV), and ongoing influenza outbreaks in poultry demonstrate both the economical relevance and the zoonotic threat for humans. Type I interferons (IFN) play an essential role in the innate host immune response against influenza viruses. The antiviral effect of IFN was first described in chicken embryos (15, 16) and later confirmed in many other species. Studies of mice revealed that the IFN-induced myxovirus resistance protein 1 (Mx1) is the main effector molecule of the IFN-induced antiviral state against FLUAV. Mouse strains carrying a functional *Mx1* gene are highly resistant to infection with influenza viruses (23). In contrast, most of the laboratory mouse strains have a defective *Mx1* gene and consequently are highly susceptible to FLUAV infection (40).

Mx proteins are large GTPases that share structural features with members of the dynamin superfamily of proteins. GTPase activity (32, 34) and the ability to form oligomers (11) are properties of Mx proteins that were identified to be important for antiviral activity. Mx proteins were described in many mammalian and nonmammalian species (1, 4, 7, 14, 27). Most species have two *Mx* genes which code for proteins that accumulate in either the nucleus or the cytoplasm of IFN-treated cells. Mouse and rat Mx1 proteins are located in the nucleus, whereas most other Mx proteins are found in the cytoplasm (as reviewed in reference 13). The question regarding the primary physiological role of Mx proteins remains unanswered. Nuclear mouse and rat Mx1 are potent inhibitors of influenza and influenza-like viruses which all replicate in the nucleus. Cytoplasmic Mx proteins such as the human MxA or bovine Mx1 not only confer antiviral activity against influenza viruses but also inhibit many unrelated viruses (2, 22, 29, 36, 38). Still other Mx proteins, such as the human MxB protein, seem to be devoid of antiviral activity (30).

In duck and chicken, only one Mx protein was identified. The lack of antiviral activity was noted for both duck Mx and chicken Mx proteins when these proteins were initially discovered (4, 7). However, more recent reports yielded conflicting results. Ko and coworkers reported that the chicken *Mx* gene is highly polymorphic, and that a single-nucleotide polymorphism affecting amino acid 631 determines antiviral activity (19, 20). Expressing these chicken Mx protein variants in the mouse fibroblast line 3T3, they observed that the Mx-631Asn variant mediated resistance against FLUAV and vesicular stomatitis virus (VSV), whereas the Mx-631Ser variant was antivirally inactive. Subsequent genetic studies revealed a considerably high frequency of the Mx-631Ser allele in distinct chicken lines (3). This observation provoked a strong interest in breeding approaches aimed at enhancing the frequency of the Mx-631Asn allele to obtain chicken lines with enhanced influenza resistance. However, FLUAV infection experiments with chickens of defined Mx-631 genotypes failed to show a correlation between susceptibility and Mx isoform (39). Furthermore, using chicken embryo fibroblasts (CEF) from different chicken lines with Mx-631Ser or Mx-631Asn as well as human HEK293T cells expressing the Mx-631Asn isoform, Benfield and coworkers were not able to confirm the proposed antiviral activity of the Mx-631Asn variant toward different FLUAV strains (5).

The aim of this study was to investigate the role of the 631 isoforms of the chicken Mx protein in IFN-mediated antiviral

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activity in chicken cells and embryos using a highly efficient retroviral expression system. This experimental setup should provide all putative species-specific cofactors required for the proper action of chicken Mx proteins. However, no protective effect of either the Mx-631Asn or the Mx-631Ser isoform was detected *in vitro* or *in vivo*. Further, the short interfering RNA (siRNA)-mediated knockdown of *Mx* gene expression did not influence the quality of the IFN-induced antiviral state against FLUAV in chicken cells. Finally, we found that unlike Mx proteins of mammalian origin, Mx protein of chickens seems to lack GTPase activity, which might explain the lack of biological activity.

MATERIALS AND METHODS

Animals. Fertilized eggs of White Leghorn chickens (LSL) were obtained from Lohmann Tierzucht GmbH, Cuxhaven, Germany, and fertilized eggs of the endogenous retrovirus-free chicken line 0 were obtained from the Institute for Animal Health, Compton, United Kingdom. Chicken embryos were incubated at 37.8°C and 55% humidity and rotated four times a day.

Cells. Chicken embryo fibroblasts (CEF) were prepared from 11-day-old embryos by trypsin digestion. CEF were maintained in Iscove's basal medium containing 5% fetal bovine serum, 2% chicken serum, and 1% penicillin-streptomycin. CEF cultures were used between passages 3 and 10. To obtain CEF which are homozygous for either the 631-Ser or 631-Asn Mx genotype, hens and roosters with the heterozygous Mx-631 genotype were mated. Eggs from this mating were used to prepare CEF cultures, and the Mx genotype of these cultures was determined as described previously (28). Madine-Darby canine kidney (MDCK) and human HEK293T cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI) containing 10% fetal bovine serum and 1% penicillin-streptomycin.

Viruses. The following virus strains were used: highly pathogenic avian influenza A virus (HPAIV) strains A/Cygnus cygnus/Germany/R65/2006 H5N1 (designated R65), A/FPV/Rostock/34 H7N1 (designated FPV Rostock), and A/Seal/Massachussetts/1/80 H7N7 (designated SC35). Work with HPAIV was performed in biosafety level 3 (BSL3) facilities. Lowly pathogenic strains A/Turkey/Ontario/6118/1968 H8N4 (designated LPAI84; avian origin) and A/WSN/1933 H1N1 (designated WSN33; human origin) were used. Virus titers are indicated as PFU for plaque developing viruses or focus-forming units (FFU) for viruses which were quantified by immunostaining.

Plasmids. 5′ NotI/ClaI and 3′ ClaI/XhoI restriction sites were amplified by PCR on chMx-631Ser and chMx-631Asn using forward primer 5′ GACAGAGCGGCC GCATCGATATGAACAATCCATGGTCCAAC 3′ and reverse primer 5′ GACA GACTCGAGATCGATCTACAGAGACTTAAAGTCTACCAGG 3′. PCR products were cut with NotI and XhoI and ligated into pCAGGS.MCS(XmaI-). pCAGGS.MCS(XmaI-)-chMx-631Ser and pCAGGS.MCS(XmaI-)-chMx-631Asn were cut by ClaI, and the two chMx isoforms were purified by agarose gel extraction. chMx-631Ser and chMx-631Asn then were ligated into the unique ClaI restriction site of RCAS(BP)A (designated RCAS). ClaI restriction sites were amplified by PCR on muMx1 using forward primer 5′ TATATCGATATGGATTCTGTGAAT AATC 3′ and reverse primer 5′ TATATCGATTTAATCGGAGAATTTGGCA 3′. The PCR product was cut with ClaI, and muMx1 was ligated into RCAS. ClaI restriction sites were amplified by PCR on huMxA using forward primer 5′ CATC ATCGATTATGGTTGTTTCCGAAGTGGAC 3′ and reverse primer 5′ CAACA TCGATTTAACCGGGGAACTGGGCAAG 3′. The PCR product was cut with ClaI and ligated into RCAS. To clone chMx-631Ser and chMx-631Asn in PCR3(HA)-Flag chMx, SalI restriction sites were amplified by PCR using forward primer 5′ GACGAGTCGACATGAACAATCCATGGTCC 3′ and reverse primer 5′ GTCTGTCGACCTACAGAGACTTAAAGTC 3′. PCR products were cut with SalI and ligated into the unique SalI restriction site of PCR3(HA)-Flag.

Generating chMx-631Asn. Oligonucleotides 5′-CACTGGAGCAAATAAAC GCCTGAGCAATCAGATTC-3′ and 5′-GCTCAGGCGTTTATTTGCTCCAG TGAAATAGGCC-3′ were used to create plasmid chMx-631Asn from chMx-631Ser. Mutagenesis was performed according to the manufacturer's instructions by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Expression of RCAS-based proteins and *in vivo* gene transfer. The RCAS-based plasmids were used to transfect line 0 CEF by calcium phosphate precipitation (18). CEF were passaged 24 h after transfection and then every 2 to 3 days. Since RCAS is a replication-competent retroviral vector, transfected cells produce productive and infective RCAS virus, and within 4 days of transfection essentially all CEF in culture expressed the retrovirally transduced protein(s). For the *in vivo* expression of RCAS-coded proteins, transfected CEF were injected into the yolk sac of fertilized eggs on the third day of incubation, delivering 10^6 virus-producing CEF per egg in an approximately 100-μl culture medium through a 21-gauge needle and a small hole drilled in the shell. Holes were sealed with liquid paraffin before eggs were returned to the incubator.

Interferon treatment of cells and embryos. Chicken IFN-α was expressed and purified from *Escherichia coli* as described previously (37). Cells were stimulated 12 h prior to infection with 500 U/ml IFN-α. For infection experiments after siRNA transfection, CEF were stimulated for 4 h with IFN-α. Embryos were checked for life signs at embryonic day 10 (ED10), and afterwards 1.5×10^5 U of IFN-α in 100 μl phosphate-buffered saline (PBS) was injected in the allantoic fluid. Eggs were sealed with paraffin wax.

Infection of cells and embryos. Monolayers of CEF and MDCK cells were infected with 50 μl per well for 96-well plates or 1 ml per well for 6-well plates of the indicated virus dilution in PBS containing 0.5% bovine serum albumin (BSA). After 1 h of incubation at room temperature, virus was removed and 2 ml of Avicel overlay medium was added to each of six wells as described before (26). For virus titration in 96-well plates, cells were overlaid with 200 μl culture medium. The incubation time depended on the virus used. Embryos were checked for life signs at ED11, and afterwards 1×10^4 FFU/ml virus in 100 μl PBS containing 0.5% BSA were injected in the allantoic fluid. Eggs were sealed with paraffin wax.

Immunostaining and immunofluorescence. After the incubation of infected cells at 37°C in 5% CO_2 to allow plaque formation, cells were fixed, permeabilized, and stained with a polyclonal antibody specific for influenza A virus for 1 h, followed by 1 h of incubation with a peroxidase-labeled anti-rabbit antibody (Jackson ImmunoResearch, Newmarket, United Kingdom) or an Alexa 488-labeled anti-rabbit antibody (Invitrogen, Darmstadt, Germany). To visualize the horseradish peroxidase (HRP)-labeled antibody, cells were incubated with the peroxidase substrate Vector NovaRED (Vector, Burlingame, CA) for 10 min. The titration of LPAI84 cell culture supernatant was done after incubation for 30 min with 10 μg/ml trypsin at 37°C. For the staining of RCAS-expressed Mx proteins, cells were fixed on cover slides, permeabilized with 0.5% Triton X-100 for 10 min, and stained with a monoclonal antibody specific for human MxA (M143; 1:500) (10), which cross-reacts with chicken and mouse Mx proteins. After 1 h of incubation, bound antibody was detected by incubation with an Alexa 488-labeled anti-mouse antibody (Invitrogen, Darmstadt, Germany).

Plaque assay. After the incubation of infected cells at 37°C in 5% CO_2 to allow plaque formation, the overlay medium was removed, and cells were fixed and stained with crystal violet.

Western blotting. For Western blotting, CEF were lysed in 1× Laemmli buffer and heated for 5 min at 95°C. Samples were separated by SDS-PAGE. Mx protein was detected by a monoclonal anti-human MxA antibody (M143), followed by a biotinylated donkey anti-mouse-IgG antibody (Jackson ImmunoResearch, Newmarket, United Kingdom), streptavidin-HRP (Jackson ImmunoResearch, Newmarket, United Kingdom), and ECL substrate (GE Healthcare, Freiburg, Germany) before being exposed to an X-ray film. Chicken β-actin was detected by a monoclonal β-actin antibody (BioVision, San Francisco, CA).

RT-PCR. Pieces of heart tissue were extracted, immediately snap-frozen in liquid nitrogen, and stored at −80°C until further processing. For RNA isolation, peqGOLD TriFast (Peqlab, Erlangen, Germany) was used according to the manufacturer's protocol. Genomic DNA elimination and reverse transcription were performed using DNase and a RevertAid H Minus first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) as described by the manufacturer. Primers for reverse transcription-PCR (RT-PCR) were designed using PerlPrimer software (25) and were obtained from Eurofins MWG Operon (Ebersberg, Germany). Primers specific for chMx were forward primer 5′-GGA GCAAGTAAACGCCTGAG-3′ and reverse primer 5′-AGGTTGCTGCTAAT GGAGGA-3′. Primers specific for muMx1 were forward primer 5′-CTCCTAT GATGACATCGAAG-3′ and reverse primer 5′-GATCCTTGAAGAAGACTT GC-3′. Primers specific for huMxA were forward primer 5′-GGCTTGCTTTCA CAGATGTTTC-3′ and reverse primer 5′-TGGCCTTCTGAAGCTGCTG-3′.

RNA interference in CEF. siRNAchMx1 (5′-GAUAUACCAACUUCUCUU U-3′) and siRNA-chMx2 (5′-UCUAGGUUGGAACGUAACU-3′), specific for chMx, were designed as described previously (33). As a nontarget siRNA, a commercial siRNA specific for enhanced green fluorescent protein (EGFP) (5′-CCGAUGCAGGUCCUCGCGUGG-3′) was used. All siRNAs were synthesized by Eurofins MWG Operon (Ebersberg, Germany). A total of 1×10^5 CEF (line 0) were reverse transfected with 50 nM siRNA using 3 μl Lipofectamine RNAiMAX (Invitrogen, Darmstadt, Germany) according to the manufacturer's

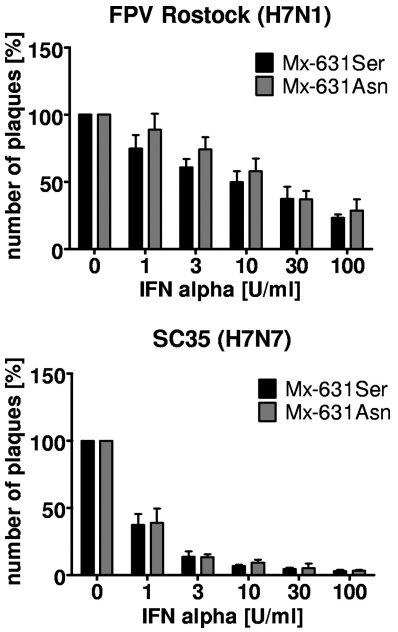

FIG. 1. No difference in influenza virus susceptibility in primary CEF with defined 631 chMx genotype. CEF from LSL chickens crossbred to be homozygous for serine (Ser) or asparagine (Asn) at amino acid position 631 in the chicken Mx protein were stimulated for 12 h with the indicated amounts of IFN-α. The CEF were infected subsequently with 100 PFU/ml of FPV Rostock or SC35 and analyzed by plaque assays for their ability to inhibit virus multiplication. Means and standard deviations from three independent experiments are shown.

instructions. Forty-eight h after transfection, cells were stimulated with IFN-α for 4 h and subsequently infected with WSN33 as described above.

Expression and purification of Flag-tagged Mx protein. Flag-Mx constructs were transfected in HEK293T cells and incubated for 48 h at 37°C in humidified 5% CO_2. After the incubation, cells were lysed and purified by anti-Flag M2 affinity gel chromatography (Sigma, St. Louis, MO) according to the manufacturer's instructions.

GTPase assay. GTPase assays were performed with equal amounts of the different Mx proteins in GTPase assay buffer (13 nM [α-^{32}P]GTP [PerkinElmer LAS, Germany], 20 mM HEPES, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 100 nM AMP-PNP) at 37°C. At various times, the reaction was stopped by adding an equal volume of a stop solution containing 2 mM EDTA and 0.5% SDS. Samples were spotted onto polyethyleneimine-cellulose thin-layer chromatography plates and resolved in running buffer (1 M acetic acid, 1 M LiCl). The plates were exposed to X-ray films. Signals were quantified as described previously (34).

RESULTS

Primary CEF with defined Mx-631 polymorphism show no difference in FLUAV susceptibility. To investigate the influence of the 631Asn/Ser polymorphism of the chicken Mx protein, primary CEF were generated from individual embryos that were homozygous at amino acid position 631 for asparagine (631-Asn) or serine (631-Ser). Five individual CEF cultures per group were pretreated with different amounts of IFN-α to induce Mx expression and subsequently infected with 100 PFU of FPV Rostock or SC35. As expected, plaque assays with these IFN-stimulated cultures revealed a dose-dependent antiviral effect of IFN pretreatment for both viruses (Fig. 1). Interestingly, protection against SC35 was far more effective than that against FPV Rostock, with almost the complete inhibition of infection in cultures treated with 100 U/ml of IFN. Reduced numbers of SC35 plaques were observed after treatment with only 3 U/ml of IFN-α. In cultures infected with FPV Rostock, only the highest IFN concentration reduced plaque numbers below 30% of untreated cells (Fig. 1). Importantly, the observed IFN effects were independent of the Mx-631 genotype. CEF lines of both *Mx* genotypes were susceptible to infection with FLUAV without prior IFN-α treatment and could be protected by stimulation with IFN-α regardless of whether chMx-631Asn or chMx-631Ser was expressed.

No inhibition of replication of lowly and highly pathogenic influenza virus strains by overexpression of either chicken Mx isoform in CEF. Although the parental chicken lines used for generating the primary CEF described above were of the same genetic background, the possibility could not be excluded that these lines possess *Mx* genes carrying additional polymorphisms besides 631-Asn/Ser. To exclude a potential influence of additional polymorphisms, a plasmid encoding chMx-631Ser which was previously described to lack antiviral activity (7) was used to generate isogenic Mx-631Asn by site-directed mutagenesis. Since mammalian cells might lack putative species-specific cofactors, we decided to express and analyze the newly generated chMx-631Asn and chMx-631Ser constructs in chicken cells. To do this, we used the well-characterized retroviral vector system RCAS (replication-competent avian sarcoma leukosis virus long terminal repeat [LTR] with a Splice acceptor) to transduce CEF of line 0 chickens. The RCAS system allows for the stable expression of the protein of interest in nearly 100% of the cells in culture. Mx-631Asn and Mx-631Ser isoforms were cloned into RCAS. Mouse Mx1 (muMx1) and human MxA (huMxA), which are known for their antiviral activity toward influenza A virus infection (12, 30), served as controls.

The expression of the different Mx proteins in line 0 CEF was controlled by immunostaining at 72 h posttransfection with the indicated RCAS-Mx constructs, a time when typically nearly all cells of the culture were infected (Fig. 2a). No Mx staining was detected in cells transfected with the empty RCAS vector. For both chMx isoforms and huMxA, clear staining was detected in the cytoplasm, whereas muMx1 accumulated in the nucleus. The staining of mouse Mx1 and human MxA revealed bright distinguished dots, whereas the staining of Mx-631Asn and Mx-631Ser was substantially weaker, less uniform, and more diffuse. This might indicate a lower degree of the oligomerization of the chicken Mx proteins. Alternatively, this effect could be caused by the lower affinity of the cross-reacting antibody for chicken Mx compared to that for mouse Mx1 and human MxA.

Analysis by Western blotting showed that RCAS-transduced cells contained considerably larger amounts of Mx proteins than cells in which endogenous Mx expression was induced by IFN (Fig. 2b). No Mx-specific bands were detected in either nonstimulated control cells or in cells transfected with empty RCAS vector. Cells with the confirmed expression of the different Mx proteins then were infected with different lowly pathogenic (WSN33, LPAI84) and highly pathogenic (R65, FPV Rostock) influenza strains. Irrespective of the virus strain used, plaque numbers in IFN-α-stimulated as well as RCAS-muMx1- and RCAS-huMxA-overexpressing CEF were strongly reduced compared to numbers for untreated controls or empty vector controls (Fig. 2c). Hence,

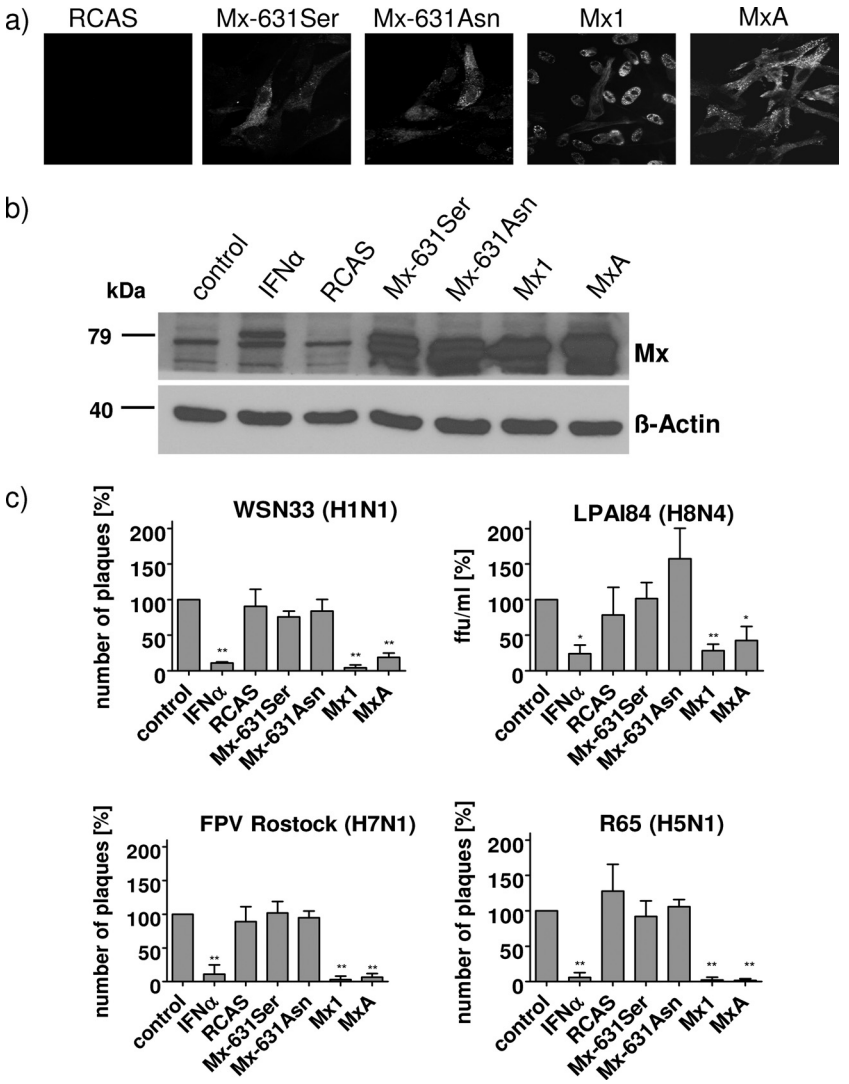

FIG. 2. Chicken Mx proteins expressed in chicken cells lack antiviral activity. (a) Line 0 CEF were transfected with the indicated RCAS constructs. At 96 h posttransfection, cells were fixed and stained with a cross-reacting monoclonal antibody against huMxA. (b) Line 0 CEF were transfected with the indicated RCAS constructs or stimulated with IFN-α for 12 h. Cells (1×10^7) were lysed, and Mx expression was determined by Western blotting. Mx has a molecular mass of approximately 79 kDa and represents the topmost band. Chicken β-actin served as a loading control. (c) Line 0 CEF were transfected with the indicated RCAS constructs or stimulated with 500 U/ml of IFN-α for 12 h prior to infection with 100 PFU/ml of the indicated viruses. For LPAI84, virus titers in the cell culture supernatant at 24 h postinfection were determined using MDCK cells. WSN33 plaques were visualized by immunostaining. Plaques of R65 and FPV Rostock were detected by crystal violet staining. Plaque numbers and viral titers of untreated control cultures were set to 100%. Significance was calculated by Student's *t* test (**, $P \leq 0.01$). Means and standard deviations from three independent experiments are shown.

pretreatment with IFN-α as well as the expression of mammalian Mx proteins conferred resistance to various influenza A viruses in these primary chicken cells. However, no reduction of the plaque numbers was observed in CEF overexpressing the Mx-631Ser or Mx-631Asn variant of chicken Mx protein, demonstrating that both chicken Mx isoforms are unable to confer antiviral activity (Fig. 2c). Consistently with earlier experiments (30), we found that the muMx1 protein inhibited influenza virus multiplication slightly more strongly than huMxA.

Chicken embryos overexpressing Mx are not protected against influenza A virus infection. The described *in vitro* experiments suggested that the chicken Mx isoforms 631Asn and 631Ser do not confer antiviral activity. However, these experiments are not necessarily conclusive, as they were performed in embryo fibroblasts which might lack necessary cofactors and which certainly are not representative for the situation in a complex organism. Hence, we decided to perform additional *in vivo* studies. Since knockout and transgene technologies for chickens are still extremely inefficient, we applied the retroviral RCAS vector system for gene transfer in chicken embryos. RCAS-Mx-infected line 0 CEF were injected into LSL embryos on day 3 of incubation (ED3). The injected cells continue to produce infectious RCAS virus which should, in turn, infect cells of the developing embryo. The integration of the RCAS vector into the host cell genome should lead to transgene expression under the control of the viral LTR.

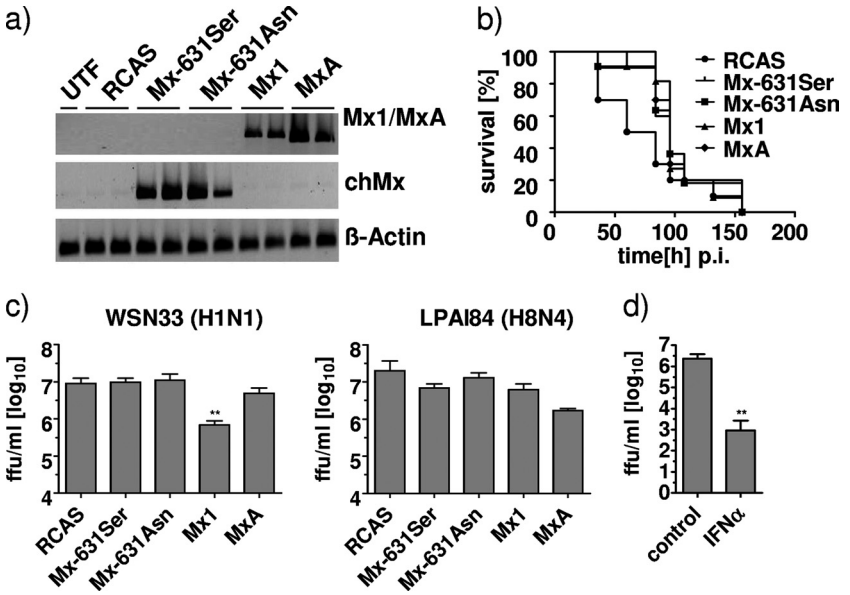

FIG. 3. IFN-α and mammalian Mx proteins but not chicken Mx mediate protection against FLUAV in chicken embryos. At ED3, chicken embryos were treated with CEF producing the indicated RCAS viruses. (a) On ED11, RNA was isolated from hearts and analyzed for Mx expression by RT-PCR with primers specific for the indicated Mx genes. Chicken β-actin served as an RNA quality control. UTF, untransfected control cells. (b and c) RCAS-transduced embryos were infected on ED11 with 1,000 FFU of WSN33 or LPAI84. (b) Survival of RCAS-treated embryos after infection with LPAI84 ($n \geq 10$ per group). (c) At 24 h postinfection, viral load in the allantoic fluid was determined by titration on MDCK cells. Means and standard deviations from at least seven embryos per group are shown. (d) ED10 chicken embryos were stimulated with 1.5×10^5 U of IFN-α in 100 μl PBS 12 h prior to and at the time of infection. The embryos were infected with 1,000 FFU of WSN33, and viral load in the allantoic fluid was analyzed 24 h postinfection by titration on MDCK cells. Means and standard deviations from at least 10 embryos per group are shown. (c and d) Significance was calculated by Student's *t* test (**, $P \leq 0.01$).

The expression of Mx isoforms in ED11 embryos was analyzed by RT-PCR with primers specific for chicken Mx, mouse Mx1, or human MxA. Heart tissue was used for RNA extraction, because high levels of RCAS-based transgene expression typically can be found in this tissue (21). A strong signal for chicken Mx was detected in embryos transduced with RCAS-chMx-631Ser or RCAS-chMx-631Asn but not in untreated embryos or embryos transduced with empty vector. Transcripts for mouse Mx1 and human MxA were clearly detected in embryos transduced with RCAS-muMx1 or RCAS-huMxA but not in control embryos (Fig. 3a).

Chicken embryos expressing the various RCAS-Mx constructs were infected with 1,000 FFU of WSN33 or LPAI84 on ED11. Survival studies revealed no difference between embryos expressing Mx1, MxA, Mx-631Asn, Mx-631Ser, or empty RCAS vector. All embryos died within 3 days when challenged with WSN33 (data not shown) or 6 days when challenged with LPAI84 (Fig. 3b). It is well known that RCAS-mediated gene transfer does not lead to transgene expression in every cell of the embryo but rather results in mosaic embryos in which a small fraction of cells (mainly endothelial cells) express the transgene (21). Hence, the antiviral effect of the different Mx proteins might not have been strong enough to mediate the survival of the embryo. Nevertheless, the RCAS-mediated expression of Mx still could have reduced viral titers early after infection. To address this possibility, allantoic fluid of infected embryos was harvested at 24 h postinfection, a time shortly before virus titers reach a plateau (data not shown), and virus load was determined on MDCK cells. No reduced virus yields were measured in WSN33-infected embryos expressing human MxA or the two different chicken Mx isoforms. However, a statistically significant inhibitory effect on virus replication was detected in mouse Mx1-expressing chicken embryos. Virus titers in allantoic fluids of muMx1-expressing eggs were more than 10-fold lower than those of control embryos expressing empty RCAS (Fig. 3c). In embryos infected with lowly pathogenic FLUAV strain LPAI84, no such differences were observed and virus yields in eggs expressing the various Mx proteins were not statistically significantly different (Fig. 3c).

For comparison, we determined the antiviral effect of IFN-α treatment *in ovo.* LSL embryos were treated with 1.5×10^5 U of IFN-α at 12 h prior to and at the time of infection with 1,000 FFU of WSN33, and viral titers in the allantoic fluid were analyzed at 24 h postinfection. Under these conditions, we observed a 1,000-fold reduction of virus growth compared to that of embryos treated with medium only (Fig. 3d). The comparably low efficiency of Mx proteins with known antiviral activity in this system probably can be attributed to the relatively small number of transgene-expressing cells. Nevertheless, the fact that the chicken Mx proteins were clearly less effective than mouse Mx1 in this experimental setup further supports the view that the chicken Mx proteins do not play an important role in IFN-mediated resistance against FLUAV.

IFN-mediated antiviral effects against FLUAV are Mx independent in chicken cells. Since IFN stimulation induces Mx in CEF and chicken embryos, we performed siRNA-mediated knockdown experiments to determine to what extent Mx contributes to the influenza-specific antiviral effect of IFN. Two siRNA molecules designed to target chicken Mx (siRNAchMx1/siRNAchMx2) and a nontarget siRNA were

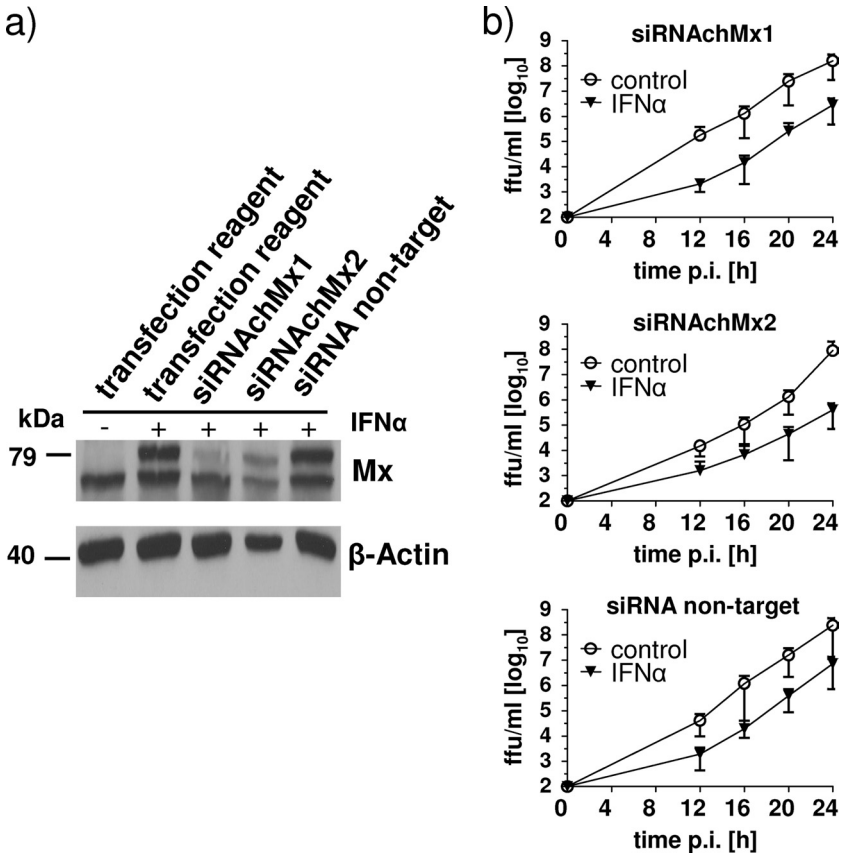

FIG. 4. IFN-α-mediated protection of CEF against FLUAV is independent of Mx. Line 0 CEF were transfected with two different siRNAs specific for chMx (siRNAchMx1 or siRNAchMx2) or a nontarget siRNA as a control. (a) Efficiency of the knockdown was measured by Western blotting. (b) Line 0 CEF with siRNA-mediated Mx knockdown were stimulated with 500 U/ml of IFN-α and infected with WSN33. The viral load in IFN-α-stimulated and control culture supernatants was determined on MDCK cells at the indicated times. Means and standard deviations from three independent experiments are shown.

transfected into line 0 CEF. Cells treated with Mx-specific siRNA molecules showed the reduced expression of Mx after stimulation with IFN-α compared to that of control cells treated with the nontarget siRNA (Fig. 4a). To analyze the effect of the Mx knockdown on virus resistance, siRNA-treated cells were stimulated with IFN-α before infection with WSN33. At different times postinfection, culture supernatant was harvested and viral titers were determined on MDCK cells. At each point of time, analyzed virus growth in IFN-α-treated cultures was reduced 100 to 1,000-fold compared to that of control cultures that were not stimulated with IFN, irrespective of whether Mx was depleted by siRNA or not (Fig. 4b), demonstrating that Mx is not required for the antiviral activity of IFN-α against FLUAV in chicken cells.

Chicken Mx protein lacks GTPase activity. GTPase activity is important for the antiviral activity of human MxA and mouse Mx1 (31). To determine if chicken Mx proteins also exhibit such enzymatic activity, plasmid constructs encoding N-terminally Flag-tagged Mx-631Asn and Mx-631Ser were expressed in HEK293T cells and purified by anti-Flag affinity chromatography (Fig. 5a). We used this eukaryotic expression system to generate recombinant chicken Mx protein, because prokaryotic expression, which worked very well for human MxA, resulted in very low yields of largely fragmented chicken Mx protein (data not shown). Human MxA and mutant MxA-T103A, which lacks GTPase activity (32), were expressed and purified in the same manner. Equal amounts of protein, as determined by SDS-PAGE, were used to analyze GTPase activity. As expected, purified MxA hydrolyzed GTP very efficiently, whereas purified MxA-T103A did not. Interestingly, samples containing purified 631Asn or 631Ser isoforms of chicken Mx hydrolyzed GTP at a rate as low as that of the GTPase-inactive MxA mutant protein (Fig. 5b). To evaluate the possibility that the observed minimal conversion of GTP by chicken Mx is due to specific activity, we generated a plasmid construct that codes for a Flag-tagged mutant form of chicken Mx (T147A) in which threonine 147 is mutated to alanine. T147 in chicken Mx is located in a position analogous to T103 in MxA and therefore is believed to similarly participate in GTP hydrolysis. When used at similar concentrations in GTPase assays, purified chMx-T147A showed background GTP hydrolysis comparable to that of purified chicken Mx proteins with no mutations in the GTPase motif, indicating that the low rate of GTP hydrolysis in our preparations most likely is due to contaminating nucleases rather than to the specific GTPase activity of the Flag-tagged proteins (Fig. 5c).

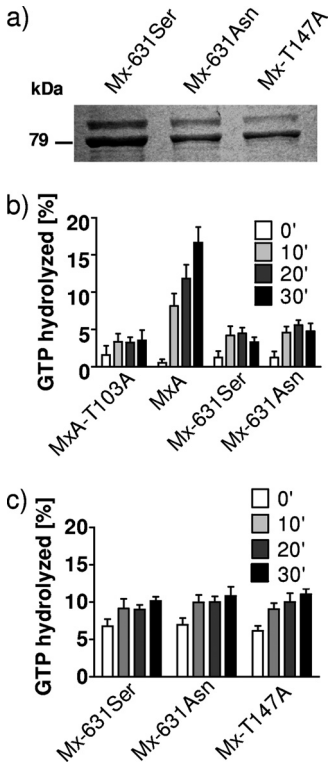

FIG. 5. Chicken Mx protein lacks GTPase activity. Mx-631Ser, Mx-631Asn, Mx-T147A, MxA, and MxA-T103A were expressed in HEK293T cells and purified by anti-M2-Flag affinity chromatography. (a) Purity and protein concentration of Mx preparations were analyzed by SDS-PAGE and Coomassie blue staining. (b and c) Equal amounts of the indicated Mx proteins were analyzed in a GTPase assay at 37°C. The reaction was stopped at the indicated time points by adding 2 mM EDTA and 0.5% SDS. Radiolabeled GDP was separated from the GTP substrate by chromatography on polyethyleneimine-cellulose-coated plates. Radioactive spots were detected by autoradiography and quantified using the MacBAS software from Fuji. Means and standard deviations from three independent experiments are shown.

DISCUSSION

The chicken Mx protein initially was found to lack antiviral activity (7). Later, Ko and coworkers reported that a single-nucleotide polymorphism at amino acid 631 is crucial for virus protection by chicken Mx (20). More recent reports from several laboratories are conflicting, some assigning and some not assigning antiviral activity to the chicken Mx protein (5, 6, 9, 19, 20, 39, 43). Thus, the question of whether Mx contributes to influenza virus resistance in the chicken has remained unanswered. By performing a series of experiments in primary CEF and fertilized chicken eggs, we evaluated the possibility that chicken Mx requires cofactors for activity that are absent in mammalian cells or established chicken cell lines. None of our experiments provided evidence in favor of the view that chicken Mx is antivirally active.

We found no difference in IFN-α-mediated protection against FLUAV in CEF homozygous for either Mx-631Asn or Mx-631Ser. This result agrees with observations from other groups who analyzed CEF (5) and chicken embryo kidney cells from genotyped animals (9). The observed difference in IFN sensitivity between FPV and SC35 possibly is due to the different histories of these two viruses. SC35, which showed a comparatively high degree of IFN sensitivity, originally was isolated from a seal before it was adapted for efficient growth in cultured chicken embryo cells (35). In contrast, FPV Rostock, which showed a low degree of IFN sensitivity, originates from an influenza outbreak in chickens and thus probably is better adapted to evade the host immune response.

We further used a retroviral gene transfer system for the overexpression of the two isoforms of chicken Mx in primary chicken cells. Neither of these proteins was able to reduce FLUAV replication in our hands, confirming earlier observations from other experimental setups by us and others (5, 7). In contrast, the expression of mouse Mx1 and human MxA resulted in a strong protective effect against lowly and highly pathogenic FLUAV from both human and avian origin. Protection by these mammalian Mx proteins was comparable to the degree of protection achieved by the treatment of the cells with IFN, demonstrating that our assay system is highly sensitive.

As studies in CEF do not necessarily mirror the situation in a complex organism, we additionally investigated the effect of RCAS-mediated Mx expression in chicken embryos. The RCAS-based transduction of embryos is known to result in mosaic tissues in which only a fraction of cells express the transgene (21). This technical limitation was probably the reason why RCAS-based expression of mammalian Mx proteins was not able to mediate prolonged survival in transduced embryos after infection with FLUAV. However, it should be noted that even under these suboptimal conditions, mouse Mx1 was able to reduce the titers of WSN33 in the allantoic fluid to a significant extent. In contrast, neither chMx-631Asn nor chMx-631Ser had a noticeable protective effect.

Unlike the case in CEF cultures, the RCAS-based expression of huMxA did not induce protection against WSN in embryos, whereas muMx1 inhibited viral replication to a significant extent. The higher antiviral potential of muMx1 previously was demonstrated in mammalian cells (30) and also is obvious from our *in vitro* experiments with WSN (Fig. 2c). The lack of antiviral activity of muMx1 and huMxA against infection of chicken embryos with a lowly pathogenic H8N4 strain is more difficult to explain. It should be noted, however, that in our *in vitro* experiments neither mammalian Mx protein was as effective against this virus as against WSN and several highly pathogenic strains (Fig. 2c). The difference in susceptibility of different influenza viruses to the antiviral action of Mx proteins also has been reported by others (8). However, the molecular basis of this discrepancy remains unexplained.

Due to the discussed shortcomings of the RCAS system, the results of our *in vivo* studies on their own certainly are not conclusive. However, the observation that none of the chMx proteins was antivirally active in the chicken embryo is in accordance with data from Sironi and coworkers (39) who infected chicken lines of distinct Mx-631 genotypes with an H7N1 FLUAV strain. These researchers found clear differences in susceptibility between the individual lines, but the enhanced resistance phenotype did not correlate with the Mx genotype.

In several distinct experimental setups, we and other groups (5, 7, 39) were not able to detect antiviral activity of any of the

chMx isotypes analyzed. This observation is in contrast to reports from Ko and coworkers (19, 20) and Ewald and coworkers (9), who described antiviral activity for the chMx-631Asn isoform. The difference between the results of Ko and coworkers (19, 20) and our current study is difficult to explain. One reason might be the different assay systems applied. Ko and coworkers (19, 20) expressed the different chMx isoforms in 3T3 mouse fibroblasts from a vector backbone with a cytomegalovirus promoter, while we used chicken cells and an avian retroviral expression system. Another explanation might be that FLUAV strains are known to differ in their sensitivity toward Mx1 and MxA (8), which also might apply for chMx proteins. Hence, the discrepancy in terms of antiviral activity might at least in part be due to the use of different virus strains.

Ewald and coworkers (9) used chicken lines which were selected for either the chMx-631Ser or chMx-631Asn genotype. They described an association of the Mx-631Asn allele with reduced viral shedding and morbidity in broiler chickens after experimental infection with FLUAV (9). However, compared to the drastic consequences of a nonfunctional Mx1 protein in mice (40), the impact of the Mx genotype in chickens was rather small. More importantly, it remains unclear whether the observed beneficial effect of the Mx-631Asn allele is indeed due to Mx protein action or rather represents a coincidental coselection of an unrelated resistance gene. Since Mx-631Asn also was associated with faster weight gain, higher mortality, and higher incidence of leg defects in broiler lines (24), the latter possibility seems quite likely.

Since the treatment of chicken cells with IFN-α protected reasonably well against FLUAV whereas the constitutive expression of chicken Mx was ineffective, it was of interest to determine if the IFN-mediated protection was entirely independent from Mx. We found that IFN remained effective in cells depleted of Mx by siRNA-mediated knockdown, suggesting that Mx is not an important mediator of IFN-induced FLUAV resistance in chicken cells. Wu and Chi recently reported a strong reduction of IFN-mediated protection of barramundi cells against a betanodavirus if Mx was knocked down (41), demonstrating that the siRNA approach is a valid method for examining Mx function.

Extensive studies with human MxA demonstrated that the GTPase activity of Mx is important for antiviral activity (38). Three distinct interfaces and one loop are believed to be involved in both oligomer formation and antiviral activity. The results of this study strongly suggest that both isoforms of chicken Mx lack GTPase activity. The reason for this surprising finding is unknown. Sequence alignments revealed no obvious structural differences between chicken and human Mx, and all important structures appear to be conserved (unpublished results). We noted that chicken Mx was much more difficult to purify than human MxA. Immunofluorescent staining of chicken Mx in CEF revealed a diffuse cytoplasmic staining pattern which is distinct from the more punctate patterns of human MxA and the antivirally active cytoplasmic Mx protein of barramundi fish (41, 42). These different staining patterns indicate that chicken Mx does not readily oligomerize, which could result in a less stable cellular protein pool (17) and explain the lack of antiviral activity. Thus, collectively our results support the view that IFN-induced Mx proteins do not strongly contribute to influenza virus resistance in chickens.

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