Human and Mouse Mx Proteins Inhibit Different Steps of the Influenza Virus Multiplication Cycle

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Human MxA and mouse Mx1 are interferon-induced proteins capable of inhibiting the multiplication of influenza virus. MxA protein is localized in the cytoplasm, whereas Mx1 protein accumulates in the nucleus. Taking advantage of stably transfected cell lines that constitutively express either MxA or Mx1 protein, we examined the steps at which influenza virus multiplication is blocked in IFN-treated cells has been difficult to establish, both inhibition of virus transcription and inhibition of viral mRNA translation being reported (3, 26). Influenza virus is a segmented negative-strand RNA virus with a complex replication strategy (for a review, see reference 14). The virus penetrates the host cell via receptor-mediated endocytosis after which membrane fusion takes place (for a review, see reference 17). After uncoating, the individual viral nucleocapsids migrate rapidly to the cell nucleus (18), where the parental viral genome is transcribed into mRNAs by the virion-associated RNA-dependent RNA polymerase, a process known as primary transcription. Influenza virus mRNA synthesis is dependent on mRNA caps that are recruited from cellular pre-mRNAs (25). Genome replication also takes place in the nucleus of infected cells (28) and is initiated after translation of the primary viral mRNAs and transport of newly synthesized viral proteins to the cell nucleus (14). Viral RNAs of negative polarity complexed with viral proteins are exported to the cytoplasm and eventually packaged. The mature virions bud from plasma membranes of the host cell.

In mice, the antiviral state induced by IFNs against influenza virus is controlled by the host gene, Mx1 (8, 9, 29). The IFN-induced Mx gene product (11) is a nuclear protein (7) that is sufficient to confer selective resistance to influenza virus (23, 24, 32). Comparing IFN-treated cells carrying or lacking functional Mx genes, we and others have previously shown that Mx1 does not interfere with host cell penetration or uncoating of influenza virus (10, 20). Furthermore, transport of parental influenza virus nucleocapsids to the host cell nucleus was not inhibited by Mx1 (4). However, further analysis of the precise step inhibited by Mx1 produced conflicting results. Krug and coworkers (15) reported that Mx1 inhibited influenza virus mRNA synthesis in IFN-treated cells. Others concluded from similar studies that Mx1 inhibited translation of influenza virus mRNAs (20).

MxA is an IFN-induced cytoplasmic protein of humans that confers resistance to influenza virus and vesicular stomatitis virus (VSV) (1, 23, 24, 31). We recently showed that MxA inhibits mRNA synthesis of VSV (33), but the mode of MxA action toward influenza virus remains unclear.

To determine the influenza virus multiplication steps blocked by human MxA and mouse Mx1 proteins, we took advantage of permanently transfected 3T3 cells that express high levels of the different Mx proteins in a constitutive manner (24). We show that mouse Mx1 interfered with influenza virus mRNA synthesis. Human MxA inhibits a step that follows primary transcription but precedes amplification of the influenza virus genome.

Infected cells expressing Mx1 or MxA contain reduced levels of influenza virus proteins. We previously demonstrated that permanently transfected Swiss 3T3 cells constitutively expressing human MxA or mouse Mx1 protein acquired resistance to influenza virus infection. In contrast, cells expressing human MxB protein remained fully susceptible to influenza virus (24). We have now determined the levels of influenza virus proteins in infected cells expressing the different Mx proteins. Monolayers of several independent cell clones expressing Mx1, MxA, or MxB protein (12, 23a, 24) were infected with 3 PFU of influenza A virus FPV-B (13) per cell for 30 min at 25°C (24). The virus inoculum was removed by two washings with phosphate-buffered saline, and the cells were further incubated in growth medium at 37°C. At 5 h postinfection, the cells were harvested and protein extracts were prepared by lysing the cells in protein

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We simultaneously measured the accumulation of primary viral transcripts (Fig. 2A) and total viral RNAs (Fig. 2B) in a panel of influenza virus-infected cell clones expressing either Mx1 or MxA. Two parallel cultures were prepared from each clonal cell line. One culture was treated with 50 μg of CHX per ml for 45 min before infection with influenza virus. This concentration of CHX, which caused minimal cytotoxic effects and inhibited protein synthesis of these cell clones by more than 98% (33), was maintained in the culture medium throughout the experiment. The second culture was kept in medium lacking CHX. Both cultures were infected with 3 PFU of influenza virus FPV-B per cell. At 3 h postinfection, the CHX-treated cultures were harvested. The infected cultures kept in CHX-free medium were harvested at 4 h postinfection. Total cellular RNA was prepared from the cultures according to the procedure of Chomczynski and Sacchi (6), and samples were separated in 1.2% agarose–formaldehyde gels and subjected to Northern (RNA) blot analysis (32). To distinguish between viral primary transcripts and genomic RNA from the parental virion, we hybridized the blots with radiolabeled in vitro-transcribed negative-sense RNA probes derived from seven of the eight influenza virus segments (Fig. 2A). The cDNA fragments coding for HA, NP, M, and NS of influenza virus A/FPV/R/34 were derived from plasmids pFPV-HA, pFPV-NP, pFPV-M, and pFPV-NS (kindly provided by C. Scholtissek). The cDNA fragments coding for the three polymerase proteins PB1, PB2, and PA of influenza virus A/PR/8/34 were derived from plasmids pAPR102, pAPR206, and pAPR303 (36). All cDNA fragments were inserted into the multiple cloning site of the vector pBS(−)Ks (Stratagene). Negative-sense RNA transcripts were synthesized in vitro, using T3 RNA polymerase (Stratagene) and [α-32P]UTP. Total viral RNA from individual viral genes (Fig. 2B) were probed with nick-translated influenza virus cDNA fragments. The intensity of the hybridization signals was quantitated by densitometric scanning of the autoradiograms.

Probes derived from the NA gene of influenza virus strains A/FPV/Rostock/34 and A/Parrot/Ulster/73 (kindly provided by C. Scholtissek) yielded only very weak hybridization signals with RNA from infected control cells (results not shown). Monitoring of primary NA mRNA synthesis was therefore not feasible.

In infected cells expressing mouse Mx1 protein, the primary transcript levels of the three polymerase genes PB1, PB2, and PA were at least 50 times lower than in infected control cells lacking Mx proteins (Fig. 2A, lanes 3 to 6). The primary transcript levels of the HA and NP genes were reduced about 15-fold, whereas the levels of the M and NS primary transcripts were only slightly reduced (3- and 2-fold, respectively). As expected from the results and from the viral protein analysis presented above, total viral RNA synthesis was strongly inhibited in cells expressing Mx1 protein (Fig. 2B). In contrast, infected cells expressing human MxA protein contained high levels of primary PB1, PB2, PA, HA, and NP transcripts, comparable to the levels in susceptible control cells (Fig. 2A, lanes 7 to 10). Surprisingly, the levels of the M and NS primary transcripts were even higher (about two- and fourfold, respectively) in cells expressing MxA than in cells lacking Mx proteins. Simultaneous monitoring of total viral RNAs (Fig. 2B) showed that all four clonal cell lines expressing MxA accumulated much less total viral RNA than did susceptible control cells. This result demonstrated that despite unhindered primary transcription, influenza virus could not replicate in MxA-expressing cells. Cells expressing MxA protein contained
significantly less viral RNA than did cells expressing Mx1 protein (Fig. 2B). This effect is due to the fact that MxA-expressing cell clones are very stable and represent more homogeneous cell populations than is true for Mx1-expressing cell clones, which always contain a low percentage of cells expressing little or no Mx1 protein.

Primary viral transcripts from cells expressing MxA are polyadenylated and can direct protein synthesis in vitro. Unhindered accumulation of primary viral transcripts in cells expressing MxA did not necessarily mean that these RNAs were functional. We therefore tested whether the primary viral RNAs from MxA-expressing cells were polyadenylated. Total cell RNAs from the experiment shown in Fig. 2A were fractionated into the poly(A)+ and poly(A)− fractions by oligo(dT)-cellulose column chromatography. Northern blots containing these RNAs were probed with radiolabeled, negative-sense RNA transcribed in vitro from influenza virus PB2 cDNA. The PB2 primary transcripts from susceptible control cells were found almost exclusively in the poly(A)+ fraction (Fig. 3, lanes 1 and 2). The PB2 primary transcripts from MxA-expressing cells were also present in the poly(A)+ fraction (Fig. 3, lanes 5 to 8), indicating that they contained a normal poly(A) tail. Similarly, the small amounts of PB2 primary transcripts detected in Mx1-expressing cells were found in the poly(A)+ RNA.

FIG. 2. Accumulation of influenza virus transcripts in infected cells expressing mouse Mx1 or human MxA protein. Two parallel cultures of the indicated 3T3 cell clones expressing either no Mx proteins (lanes 1 and 2), Mx1 (lanes 3 to 6), or MxA (lanes 7 to 10) were infected with 3 PFU of influenza virus FPV-B per cell. To measure primary viral genome transcription (A), infection was carried out for 3 h in the presence of 50 μg of CHX per ml. To measure overall viral RNA synthesis (B), infection was carried out for 4 h in the absence of CHX. Total RNA was prepared from each culture; samples were separated in 1.2% agarose-formaldehyde gels and subjected to Northern blot analysis. For the primary transcription experiments, 15-μg samples of the different RNAs were loaded into each lane, and the Northern blots were hybridized with radiolabeled, negative-sense RNA probes derived from the indicated influenza virus genes. The blots were exposed to X-ray films for 72 h at −70°C, using intensifying screens. For overall viral RNA analysis, 5-μg samples of the different RNAs were loaded into each lane, and the Northern blots were hybridized with radiolabeled cDNA probes of the indicated influenza virus genes. The blots were exposed to X-ray films for 2 h (M and NS), 4 h (HA and NP), or 8 h (PB1, PB2, and PA) at room temperature.

FIG. 3. Effect of MxA on the polyadenylation of influenza virus primary transcripts. The indicated cell clones expressing no Mx proteins (lanes 1 and 2), Mx1 (lanes 3 and 4), or MxA (lanes 5 to 8) were treated with 50 μg of CHX per ml and infected for 3 h with 3 PFU of influenza virus FPV-B per cell. Total RNA was prepared from each culture, and 40-μg samples were fractionated by oligo(dT)-cellulose chromatography. The poly(A)+ RNA fractions (lanes 1, 3, 5, and 7) and the poly(A)− RNA fractions (lanes 2, 4, 6, and 8) were separated in a 1.2% agarose–formaldehyde gel and subjected to Northern blot analysis. Radiolabeled negative-sense PB2 RNA was used as a hybridization probe. The positions of PB2 mRNA and cross-hybridizing 28S rRNA are indicated.
FIG. 4. Effect of MxA on the in vitro translation capacity of influenza virus primary transcripts. The indicated cell clones expressing no Mx proteins (lanes 1 to 3), Mx1 (lane 4), or MxA (lanes 5 and 6) were treated with 50 μg of CHX per ml and infected for 3 h with 3 PFU of influenza virus FPV-B per cell. Total RNA was prepared from each culture and fractionated by oligo(dT)-cellulose chromatography. Samples (0.5 μg) of poly(A)+ RNA were translated in vitro, using rabbit reticulocyte lysate and [35S]methionine. Poly(A)+ RNAs from infected control cells not treated with CHX (lane 1) and from uninfected cells (lane 2) were included as controls. The in vitro-translated viral proteins were immunoprecipitated with a mouse antiserum to influenza virus A/Turkey/England/63. The bands corresponding to the viral proteins NP, HA1, M, and NS are indicated. The relative positions of marker proteins are depicted at the left.

fraction (Fig. 3, lanes 3 and 4). The strong signal observed in all poly(A)+ RNA fractions is due to cross-hybridization of 28S rRNA with the PB2 RNA probe.

We next examined whether the viral mRNAs could direct protein synthesis in vitro. Samples of 0.5 μg of poly(A)+ RNAs were translated in vitro, using rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine. Viral proteins were then immunoprecipitated (19) with an antiserum of mice immunized with inactivated influenza virus A/Turkey/England/63. The immunoprecipitated translation products were analyzed on a 10% polyacrylamide–sodium dodecyl sulfate gel (30). Similar levels of radiolabeled NP were detected irrespective of whether the templates were mRNAs from CHX-treated control cells (Fig. 4, lane 3) or mRNAs from CHX-treated cells expressing MxA (Fig. 4, lanes 5 and 6). The M and NS proteins were produced at slightly higher levels with templates of MxA-expressing cells than with templates of control cells. Only very low levels of NP protein were observed when poly(A)+ RNA of Mx1-expressing cells was used as the template (Fig. 4, lane 4), whereas low but significant amounts of M and NS proteins were synthesized from such RNA. Thus, the relative amounts of viral proteins synthesized in vitro reflected the relative amounts of the corresponding primary transcripts in the different cell lines, suggesting that the primary influenza virus transcripts from MxA-expressing cells could be translated normally.

Primary influenza viral transcripts accumulate in the cytoplasm of MxA-expressing cells. In permissive cells, influenza virus mRNAs are efficiently transported to the cell cytoplasm (28). To test whether MxA affected the transport of viral mRNAs, we analyzed the intracellular distribution of primary influenza virus transcripts in infected cells expressing MxA. Susceptible control cells and MxA-expressing cells were infected with 3 PFU of influenza virus FPV-B per cell in the presence of CHX. At 3 h postinfection, total RNAs were prepared from the cytoplasmic and nuclear fractions of each culture, and the RNAs were analyzed by the Northern blotting technique. For the isolation of cytoplasmic and nuclear RNAs, the cells were lysed in lysis buffer (10 mM Tris–hydrochloride [pH 7.5], 10 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet P-40) for 5 min on ice. The nuclei were sedimented for 5 min at 1,500 × g, and the supernatant was designated the cytoplasmic fraction. The nuclei were washed once in lysis buffer. Sodium dodecyl sulfate and EDTA (final concentrations of 0.4% and 10 mM, respectively) were added to both fractions, and nuclear and cytoplasmic RNAs were isolated as previously described (22, 32). The purity of the nuclear and cytoplasmic fractions was monitored as described by Shapiro and coworkers (28). Radiolabeled negative-sense NP RNA was used as a hybridization probe. The primary NP transcripts were detected exclusively in the cytoplasmic fractions of both control cells and cells expressing MxA (Fig. 5). Reprobing of the membrane with radiolabeled positive-sense PB2 RNA yielded no signals, indicating that the sensitivity of this assay was not high enough to detect the genomic RNA of the parental virus (data not shown).

Conclusions. We have recently shown that constitutive expression of human MxA and mouse Mx1 protein in stably transfected mouse 3T3 cells conferred resistance to influenza virus infection (24). Taking advantage of these cell lines, we now examined which steps of the influenza virus multiplication cycle were blocked by these two proteins known to accumulate in different subcellular compartments. We show here that the nuclear mouse Mx1 protein inhibited viral mRNA synthesis, whereas the cytoplasmic human MxA protein blocked amplification of the viral genome without affecting primary transcription.

Previous studies aimed at elucidating the influenza virus multiplication steps blocked by Mx1 protein in IFN-treated cells generated conflicting results. Using IFN-induced mac-
virus genes was not uniformly inhibited by Mx1 protein. The degree of inhibition correlated with the length of the genomic segments. The levels of the longest primary transcripts coding for PB1, PB2, and PA were reduced at least 50-fold in infected cells expressing Mx1, whereas the levels of the shortest primary transcripts coding for M and NS were reduced at best 2- to 3-fold (Fig. 2A).

Mx1 protein interferes with an important step of viral multiplication, since the production of polymerase mRNAs in the cell nucleus and subsequent synthesis of polymerase proteins are absolutely essential for amplification of the viral genome. Our experiments do not allow us to distinguish between decreased stability of primary transcripts and reduced synthesis of primary transcripts. However, the observed reduction of viral RNA synthesis in the presence of Mx1 (15) argues in favor of an inhibition of the transcriptional activity of the viral polymerase by Mx1. The fact that the extent of the inhibition of RNA accumulation correlated with segment length (Fig. 2A) (15) suggests that chain elongation, rather than initiation, is the target of Mx1 action. Premature chain termination is expected to preferentially affect the transcripts of longer genes, and incomplete RNA transcripts lacking a poly(A) tail are usually very unstable. Interestingly, MxA protein has a similar inhibitory effect on transcription of the VSV genome. We found that in VSV-infected cells expressing MxA protein, the relative levels of the long primary transcripts were reduced much more dramatically than were levels of the short transcripts (33).

In contrast to Mx1, the human MxA protein has no inhibitory effect on primary transcription of influenza virus. The primary transcripts of influenza virus accumulating in infected cells expressing MxA protein were polyadenylated and able to direct protein synthesis in vitro. Furthermore, these RNAs appeared to be efficiently transported to the cytoplasm. The precise step at which influenza virus is blocked by MxA protein remains undefined. However, our results indicate that MxA protein interfered either with access of the viral mRNA to the protein synthesis machinery, with viral protein synthesis, with transport of newly synthesized viral proteins back to the cell nucleus, or with viral genome replication. Because MxA-expressing cells readily synthesized influenza virus proteins after transfection with plasmid vectors encoding influenza virus proteins (21a), protein synthesis is presumably not the MxA-sensitive step. Also, viral RNA replication appears to be an unlikely target of MxA action, since MxA is a cytoplasmic protein and thus presumably blocks a multiplication step occurring in the cytoplasm. In the light of recent findings that Mx proteins are homologous to a family of proteins involved in microtubule-dependent intracellular protein transport (2, 5, 21, 27, 34, 35), it is possible that MxA protein interferes with the intracytoplasmic transport of viral RNAs to ribosomes or with translation of newly synthesized viral proteins from the cytoplasm to the nucleus.

Although MxA and Mx1 inhibit different steps of the influenza virus multiplication cycle, the two proteins could still exert their function by a common mechanism. The differences reported here might simply stem from the fact that MxA and Mx1 are localized in different subcellular compartments. Recent experiments indicate that this might indeed be the case (37). When moved to the cell nucleus with the help of a foreign nuclear translocation signal, human Mx protein retained its activity against influenza virus. But unlike the cytoplasmic MxA protein, the nuclear form of MxA blocked primary transcription of influenza virus, thereby mimicking mouse Mx1 protein.

We recently showed that the inhibitory effect of Mx1 protein against influenza virus could be neutralized by overexpression of the three viral polymerase proteins and to a lesser extent by PB2 alone (12), suggesting that Mx1 can physically interact or functionally compete with components of the influenza virus polymerase complex, thereby blocking its catalytic function. It remains to be determined whether overexpression of proteins of the influenza virus RNA polymerase complex can also neutralize the inhibitory effect of the mutant MxA protein in the nucleus and the wild-type MxA protein in the cytoplasm.

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