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Switch in Antiviral Specificity of a GTPase upon Translocation from the Cytoplasm to the Nucleus

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The Mx2 protein of rats is a cytoplasmic GTPase that protects cells against vesicular stomatitis virus but not against influenza virus. Since vesicular stomatitis virus replicates in the cytoplasm and influenza virus replicates in the nucleus, it was possible that the antiviral specificity of rat Mx2 protein was determined solely by the protein's subcellular localization. Here, we found that, indeed, rat Mx2 protein lost its anti-vesicular stomatitis virus activity and gained anti-influenza virus activity when it was directed to the nucleus by way of a foreign nuclear-transport signal appended to its amino terminus. These data show that rat Mx2 protein possesses an antiviral activity that is revealed only when the protein is shuttled to the nucleus.

Vertebrate Mx proteins constitute a family of alpha/beta interferon-inducible large GTPases (M_r = 70,000 to 80,000) (6, 13; for a review, see reference 1). They accumulate in distinct subcellular localizations, and some of them have potent antiviral activities against specific groups of viruses. For instance, the murine Mx1 protein is essential for protection against influenza viruses (2, 19). It accumulates in the cell nucleus (4), where it interferes with the accumulation of influenza virus mRNAs (9, 16). In contrast, the human MxA protein has a dual antiviral specificity and accumulates in the cytoplasm, where it interferes with the accumulation of influenza virus proteins (16, 17) and vesicular stomatitis virus (VSV) mRNAs (20). Rats have three Mx genes which give rise to three Mx proteins, rat Mx1 (rMx1), rMx2, and rMx3 (11). Rat Mx1 is a nuclear protein that protects cells predominantly against influenza virus, and rMx2 is a cytoplasmic protein that protects cells only against VSV. The rMx3 protein is also cytoplasmic but lacks activity against either virus, even though it differs from rMx2 at only eight amino acid positions (12) and shares GTPase activity with rMx2 (8).

Thus, it appears that, in general, nuclear Mx proteins inhibit influenza virus and cytoplasmic Mx proteins, provided they are active, inhibit VSV. This raises the interesting possibility that the distinct antiviral specificities of natural Mx proteins are determined by their subcellular localization. To test this hypothesis, particular Mx proteins may be relocated to different subcellular compartments either by directly microinjecting purified proteins or by expressing modified proteins whose sequences include artificial targeting signals or alterations in their natural targeting signals. However, modifications in the sequence of a protein may not only change its targeting but independently destroy its intrinsic antiviral activity. Thus, the loss of anti-influenza virus activity of a mouse Mx1 protein truncated in its carboxyl-terminal nuclear transport signal (14, 21) was apparently not due simply to the protein's retention in the cytoplasm, since relocation to the nucleus by addition of a foreign nuclear-targeting signal did not restore activity, even though it did restore activity in two other Mx1 derivatives whose carboxyl termini have been altered in a different way (21).

Here, we directed the cytoplasmic rat Mx proteins, rMx2 and rMx3, to the nucleus by appending to their amino termini the simian virus 40 (SV40) large T nuclear-location signal. The modified proteins accumulated efficiently in the nuclei of cells in which corresponding plasmids were expressed. We found that rMx3 remained inactive against the virus even when transported into the nucleus. However, when rMx2 accumulated in the nucleus, it gained anti-influenza virus activity and lost its anti-VSV activity. The data indicate that this switch in antiviral specificity was due solely to the nuclear relocation of an otherwise unaltered protein.

To express rat Mx2 protein containing the SV40 nuclear-localization signal (7), a corresponding cDNA construct was generated by a polymerase chain reaction-based strategy that allows the insertion of specific sequences at any chosen location. As depicted in Fig. 1, the polymerase chain reaction contained an rMx2 cDNA (12) and three different oligonucleotides, ONT1, ONT2, and ONT3. ONT1 is a 50-mer that contains close to its 5′ end a *Bsm*I restriction site followed by the coding information for the initiator methionine, a dipeptide spacer, and the first seven amino acids of the nuclear-localization signal. ONT2 is a 44-mer that contains additional coding information for the nuclear transport signal and a dipeptide spacer. It anneals to 13 nucleotides at the 3′ end of ONT1 and to 15 nucleotides corresponding to codons 2 to 6 of the rMx2 cDNA. ONT3 is a 27-mer that corresponds to nucleotide positions 1917 to 1944 of the cDNA and contains a *Bsm*I site. The resulting 1,810-bp polymerase chain reaction product was subcloned after digestion with *Bsm*I, and the sequence of a particular clone between the first *Bsm*I and a unique *Hin*cII site corresponding to position 236 of the rMx2 cDNA was confirmed. The 96-bp *Bsm*I-*Hin*cII fragment of this clone was then used to substitute the 5′ end of the rMx2 cDNA up to the *Hin*cII site at nucleotide position 236. Since rMx2 and rMx3 cDNAs are identical in their open reading frames up to this *Hin*cII site, an analogous exchange was performed with the rMx3 cDNA. The resulting signal-containing Mx2 and Mx3 cDNAs were then cloned behind the SV40 early enhancer-promoter of the expression vector pSS-1 (12) to yield pSVrMx2-N and pSVrMx3-N, respectively.

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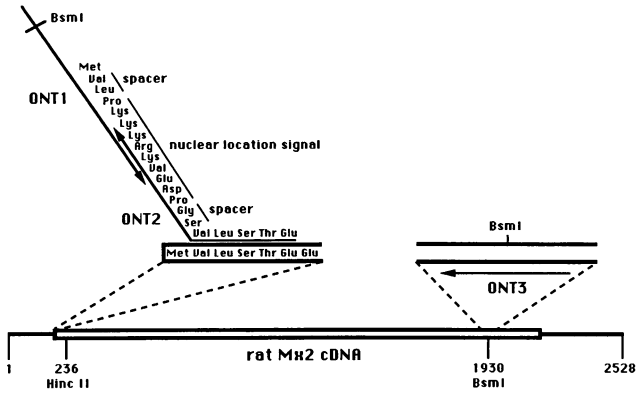

FIG. 1. Insertion of the SV40 large T nuclear-location signal into rMx2 cDNA. A polymerase chain reaction was carried out with the rat Mx2 cDNA clone pSVrMx2 and the three oligonucleotides, ONT1 (5′ AGCTCAAAGCATTCTAAAAGATGGTTCTTCCAAA AAAGAAGAGAAAGGTA 3′), ONT2 (5′ CCTCTGTGCTAAGAA CGGATCCTGGGTCTTCTACCTTTCTCTTC 3′), and ONT3 (5′ TGTCGGTAGGCATTCAGATGCTGGAAG 3′). The generated DNA fragment contains *Bsm*I sites close to both termini for convenient cloning. The encoded protein contains the 10-amino-acid-long SV40 large T nuclear-location signal together with a dipeptide spacer on either side inserted between the first and second amino acids of the rMx2 protein.

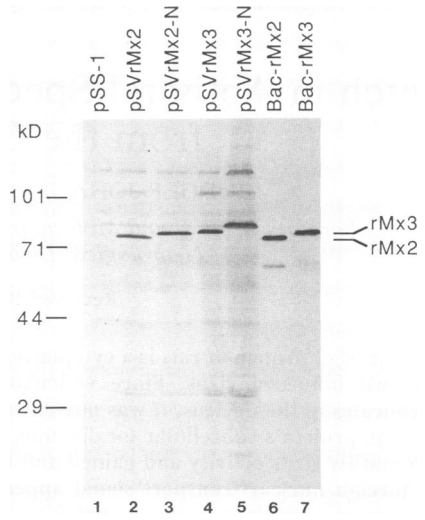

FIG. 2. Western blot analysis of rMx2-N and rMx3-N. COS cells were transfected with pSS-1 that lacks an Mx insert (lane 1), pSVrMx2 (lane 2), pSVrMx2-N (lane 3), pSVrMx3 (lane 4), and pSVrMx3-N (lane 5). Forty hours after transfection, total cell extracts were prepared and separated on a 12.5% polyacrylamide gel. The proteins were blotted onto a nylon membrane, and Mx proteins were visualized with a polyclonal rabbit anti-rMx3 antibody and an alkaline phosphatase-conjugated secondary antibody. Lanes 6 and 7, extracts of insect cells infected with rMx2 and rMx3 baculoviruses, respectively.

In order to assess whether the signal-containing expression plasmids would give rise to Mx proteins of the expected sizes and immunoreactivity, pSVrMx2-N and pSVrMx3-N were transfected into COS cells by the calcium phosphate coprecipitation method (15). As controls, plasmids pSVrMx2 and pSVrMx3, from which wild-type rMx2 and rMx3 proteins are expressed (12), were transfected into parallel cultures. Total cell lysates were prepared 40 h after transfection and subjected to Western blot (immunoblot) analysis using a polyclonal rabbit anti-rMx3 serum (8). The immunoreactive proteins were compared with rMx2 and rMx3 proteins that were expressed in insect cells after infection with corresponding recombinant baculoviruses and that comigrate with the respective authentic Mx proteins of interferon-induced rat cells (10). The results of the Western analysis are shown in Fig. 2. As expected, both wild-type proteins, rMx2 and rMx3 (Fig. 2, lanes 2 and 4, respectively), from COS cell extracts comigrated with the corresponding baculovirus Mx proteins (apparent M_rs = 75,000 and 77,000, respectively [Fig. 2, lanes 6 and 7]). Transfection with pSVrMx2-N resulted in the synthesis of an immunoreactive protein with the apparent M_r of 77,000, and transfection with pSVrMx3-N resulted in the synthesis of a protein with the apparent M_r of 79,000 (Fig. 2, lanes 3 and 5, respectively). These molecular weights are slightly higher than those of the corresponding parental proteins, as would be expected from the addition of the targeting signal. Thus, the modified proteins were apparently of full size, and they were termed rMx2-N and rMx3-N, respectively.

To determine whether the nuclear-localization signal was functional, the corresponding plasmids were transfected into COS and mouse BALB/3T3 cells or needle injected into BALB/3T3 cells. The cells were analyzed by immunofluorescent staining using monoclonal antibody 2C12 (4) as described previously (12). The results were similar irrespective of the cell type or the method used for the introduction of the plasmids. As expected, the wild-type rMx2 and rMx3 proteins were found exclusively in the cytoplasm (for BALB/3T3 cells, see Fig. 3B and C, respectively). In contrast, in a majority of cells, rMx2-N or rMx3-N proteins were found exclusively in the nuclei, as shown for single cells in Fig. 3D and E, respectively. Both proteins appeared less punctate in the nucleus than the naturally nuclear, wild-type rMx1 protein (Fig. 3A) that was expressed from a plasmid described earlier (12). In a small subfraction of either COS or BALB/3T3 cells rMx1, rMx2-N, and Mx3-N were found both in the nucleus and in the cytoplasm, and in another subfraction they were found exclusively in the cytoplasm. Such cell-to-cell variations in nuclear transport have also been reported for other systems (5).

The antiviral activities of rMx2-N and rMx3-N were analyzed in a transient-expression system, since stably transformed cells could be obtained only with rMx3 and rMx3-N and not with rMx2 or rMx2-N; it was possible that constitutive high-level expression of the latter two proteins was toxic to the cells (data not shown). Similar observations have been made with a modified, cytoplasmic mouse Mx2 protein and a human MxA protein targeted to the nucleus (22, 23). We have shown previously that microinjection of Mx expression plasmids into BALB/3T3 cells (that is, cells unable to synthesize an Mx protein of their own [18]) is a feasible method for assessing the antiviral activity of a particular Mx protein (12). Therefore, BALB/3T3 cells were injected with the respective plasmids and, 18 h later, infected with either VSV or influenza virus A/WSN at a multiplicity of infection of approximately 10. Three hours after infection with VSV or 8 h after infection with influenza virus, the cells were fixed and processed for double indirect immunofluorescent staining to monitor Mx protein expression and accumulation of viral proteins in the same cell as described previously (12). Infection with either virus did not affect the

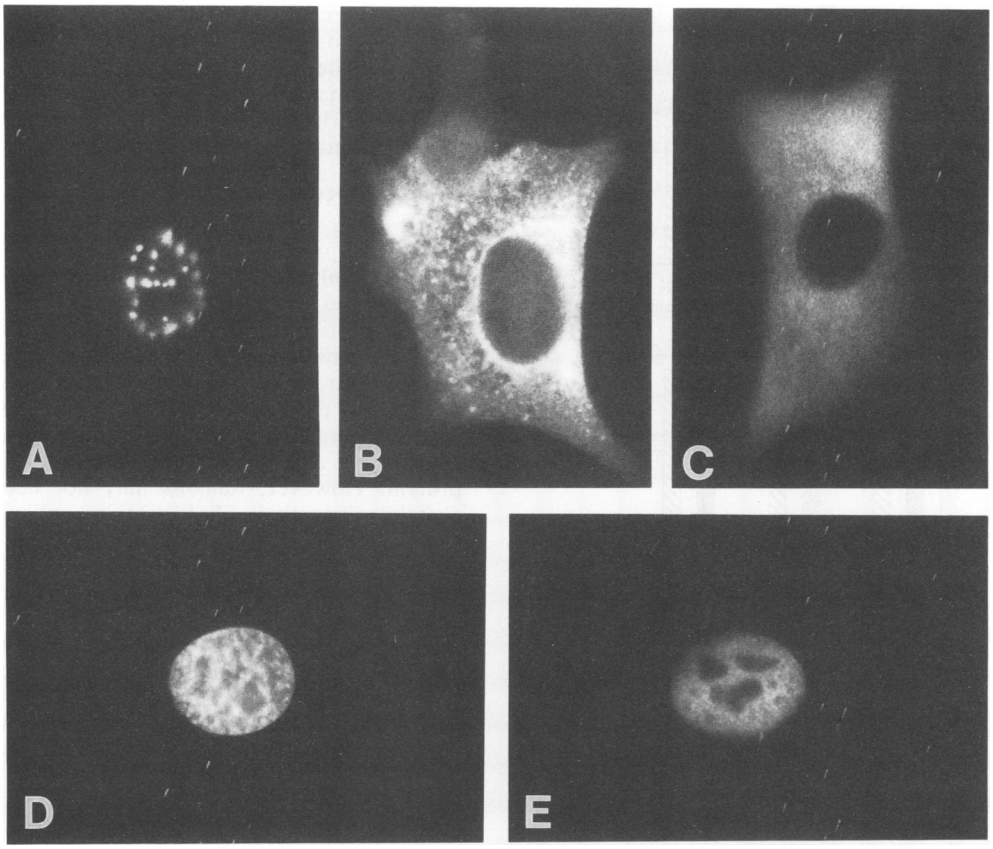

FIG. 3. Subcellular localization of signal-containing rat Mx2 and Mx3 proteins. Mouse BALB/3T3 cells that are devoid of endogenous Mx proteins were injected with pSVrMx1 (A), pSVrMx2 (B), pSVrMx3 (C), pSVrMx2-N (D), and pSVrMx3-N (E). The cells were incubated for 18 h, fixed, and stained for Mx protein with antibody 2C12 (4). The wild-type rMx1 as well as rMx2-N and rMx3-N proteins accumulate in the majority of cells only in the nucleus, whereas the wild-type rMx2 and rMx3 proteins are located in the cytoplasm.

subcellular localization of the expressed Mx proteins (data not shown).

A quantitation of the antiviral activities is shown in Fig. 4. In cells expressing the cytoplasmic rMx2 protein (Fig. 4A), the percents VSV- and influenza virus-positive cells were similar to those observed previously (12), namely, 5% in the case of VSV and 93% in the case of influenza virus. However, the results obtained with rMx2-N were distinctly different. The combined counts from two independent experiments show that 84% (58 of 70) of cells in which rMx2-N was found exclusively in the nucleus stained positive for VSV while only 16% (10 of 64) of such cells were positive for influenza virus (Fig. 4A). The percents protected cells correlated well with the apparent level of nuclear rMx2: cells with high rMx2 levels rarely stained virus positive, while cells with low levels were virus positive more frequently. It is unlikely that the switch in antiviral specificity upon nuclear translocation was due to structural changes in an antiviral domain of the modified rMx2 protein. Only 4% (1 of 28) of cells in which the protein was retained in the cytoplasm stained positive for VSV, while 80% (16 of 20) of such cells were positive for influenza virus (Fig. 4A). Thus, rMx2-N in the cytoplasm behaved like wild-type rMx2; it did not inhibit influenza virus, but it inhibited VSV. As shown in Fig. 4B, rMx3-N remained inactive against either virus, independently of where it accumulated. Therefore, this protein served as a control to exclude potential adverse side effects of high-level protein expression or the microinjection procedure. In addition, the results obtained with rMx3 lend further support to the notion that cells that are for unknown reasons unable to transport the amino-terminally modified Mx proteins efficiently into the nucleus remain in principle susceptible to viruses. Therefore, we conclude that the switch in antiviral specificity observed with rMx2-N is due solely to its relocation to the nucleus.

The switch in antiviral specificity of the wild-type rMx2 protein upon translocation into the nucleus was intriguing, since it resulted in the gain of a biologically relevant function. This is in contrast to a previous study in which the naturally cytoplasmic human MxA protein was similarly modified with a nuclear-transport signal and constitutively expressed in mouse cell lines. In the one cell line analyzed, nuclear human MxA protein retained protection against influenza virus but not against VSV. However, this result was left uninterpreted since the expression levels achieved in this cell line were very low (22). In addition, despite being expressed at higher levels, a human MxA protein with a mutation at residue 645 could not be tested for a shift in antiviral specificity for it had lost anti-VSV activity already when in the cytoplasm (22). Furthermore, various mutant mouse Mx1 proteins were inactive against both influenza virus and VSV when translocated from the nucleus to the cytoplasm (21). Thus, neither of these studies allowed the conclusion that the subcellular localization is the sole deter-

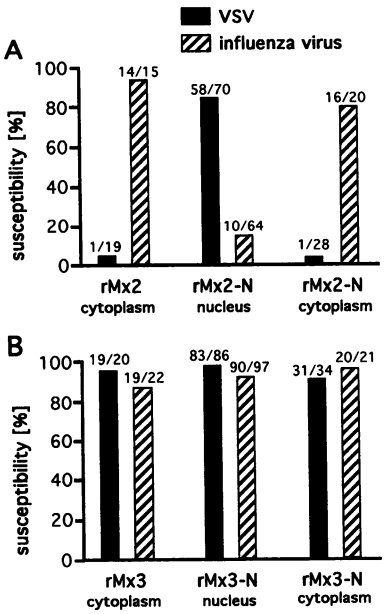

FIG. 4. Antiviral activities of cytoplasmic and nuclear rMx2 and rMx3 proteins in mouse BALB/3T3 cells. Plasmids coding for either signal-containing or wild-type rMx2 and rMx3 proteins were injected into the nuclei of Mx-negative mouse BALB/3T3 cells. Eighteen hours after injection, the cells were infected with either VSV or influenza virus A/WSN at a multiplicity of infection of 10, and later they were fixed and double labeled for rat Mx proteins (with monoclonal antibody 2C12, [4]) and virus proteins (with rabbit antisera directed against the whole virus). On the basis of the cellular compartment (nucleus or cytoplasm) in which the signal-containing Mx proteins accumulated, cells were divided into two groups, which were analyzed separately. Viral susceptibilities of cells are given as percents virus-positive cells of the total Mx protein-expressing cells. Each column represents the combined results obtained by counting cells in three to five dishes in each of two independent experiments. The total number of cells expressing virus proteins per total number of cells expressing Mx protein is indicated above each column. The infection rates of uninjected cells for VSV and influenza virus were 95 and 90%, respectively.

minant of the antiviral specificity of wild-type Mx proteins, in contrast to the results obtained with rat Mx2 protein presented above, which clearly indicate that this is the case.

The results can be explained in several ways. A simple explanation would require that the nuclear form of rMx2 protein be capable of directly interacting with distinct influenza virus proteins or nucleic acids in the nucleus and the cytoplasmic form of rMx2 be capable of doing so with distinct VSV proteins or nucleic acids in the cytoplasm. This interaction could be mediated by a single protein domain of rMx2 or, conceivably, by one domain specific for influenza virus and another one specific for VSV. However, neither of these possibilities is likely, for we are not aware of any significant similarities among the two viruses, let alone any demonstration that Mx proteins would directly bind to virus proteins, nucleocapsids, or RNAs.

The described results may also be explained by an indirect action of rMx2. Nuclear rMx2 may modify some nuclear host factor required for influenza virus replication, whereas cytoplasmic rMx2 protein may modify a cytoplasmic host factor required for VSV replication. Such host factors may be identical or closely related to each other, so that it is conceivable that they interact with similar efficiencies with rMx2 and are required, each in its respective compartment, for replication of either virus. In addition, some host factors, rather than being modified by rMx2, may themselves modify rMx2 and may modify it differently in the different subcellular compartments. In any event, an explanation in terms of an indirect mode of action of rMx2 protein (or of other Mx proteins) is attractive for two reasons: (i) the replication cycles of VSV and influenza virus are indeed influenced by host cellular factors, and (ii) Mx proteins are GTPases (6, 13), that is, proteins known for their abilities to interact with various cellular factors which they regulate or by which they are regulated (3).

It is tempting to speculate that in the course of evolution, a simple mutation that would have resulted in the transport of an ancestral cytoplasmic Mx protein into the nucleus may have given rise to the potent nuclear anti-influenza virus proteins of rodents. Interestingly, humans, for whom influenza virus is a natural pathogen, do not express a nuclear Mx protein. However, humans may not need a nuclear Mx protein, since their MxA protein, unlike rMx2, is active against influenza virus in the cytoplasm (17). But then, human MxA (or any other Mx protein) may have been maintained in evolution for reasons other than protection against viruses (1).

In conclusion, we have shown that one and the same GTPase, rMx2, has different activities, depending on where the protein accumulates. This suggests that other proteins may possess activities that go unnoticed when they are tested only in their physiological compartments.

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