Nuclear Cotransport Mechanism of Cytoplasmic Human MxB Protein*

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Interferon-α/β-inducible Mx proteins belong to the family of large GTPases and share high sequence homology with dynamin in their N-terminal GTP-binding domains. In addition, Mx proteins have a conserved C-terminal leucine zipper element that is involved in their oligomerization. Cytoplasmic human MxA protein mediates resistance to multiple RNA viruses, whereas no antiviral activity has been found for human MxB protein. We have previously shown that MxB protein exists as a nuclear 78-kDa and as a cytoplasmic 76-kDa form in interferon-α-induced human cells. Using various influenza hemagglutinin epitope-tagged MxB gene constructs in transient transfection experiments in COS-1 cells, we show that the cytoplasmic 76-kDa MxB protein forms hetero-oligomers with the nuclear 78-kDa MxB protein via the C-terminal leucine zipper element. This enables the cytoplasmic form of MxB protein to be translocated into the nucleus together with the nuclear form of MxB protein. This finding was confirmed in interferon-α-induced HEp-2 and T86G cells transfected with various MxB gene constructs. Cell fractionation studies also suggest that a considerable amount of the cytoplasmic MxB protein is also found in the nucleus. Using confocal laser microscopy, we also demonstrate that the cytoplasmic MxA and the nuclear MxB proteins do not colocalize/oligomerize with each other, and both of these proteins are retained in their specific cellular compartments.

The gene expression of intracellular Mx proteins is strictly regulated by type I interferons (IFN-α) (1–4). Human MxA as well as rodent Mx1 and Mx2 proteins have been shown to inhibit the replication of different types of negative-stranded RNA viruses, like influenza A, vesicular stomatitis, and measles viruses, as well as members of the Bunyavirus family (5–13). Mx proteins, such as human MxB protein with no demonstrable antiviral activity, have also been described (6, 11, 14, 15). Sequence data from at least eight different vertebrate species reveal several conserved features in Mx proteins. They all have a tripartite GTP-binding domain in the N-terminal third of the protein. Mx proteins can readily hydrolyze GTP with an intrinsic GTPase activity ranging from 3 to 70 min⁻¹ (15–20). The enzyme activity is a prerequisite for the antiviral activity of these proteins (7, 19). In addition to their GTP-binding elements, Mx proteins have a conserved C-terminal leucine zipper domain, which is capable of mediating their oligomerization both in vivo and in vitro (21). Both rodent Mx1 and human MxB proteins also have a specific nuclear targeting signal (3, 21–23).

We have previously shown that the human MxB protein is found both in the cell cytoplasm and nucleus, typically in a granular pattern (3). Transfection experiments in COS-1 cells of N-terminally deleted MxB constructs revealed a functional nuclear localization signal (NLS) within the first 24 N-terminal amino acids of the protein. In all the studied cell types, IFN-α induced the expression of MxB protein of two different molecular masses, namely 78- and 76-kDa forms. The 78-kDa protein represents a full-length translation product of the MxB gene with a putative NLS. Instead, the 76-kDa protein is apparently being translated from the second AUG codon of the same MxB mRNA. In primary leukocytes, the full-length NLS-containing 78-kDa protein constituted approximately 25% of the total MxB protein immunoreactivity (3).

In the present study, we show that the nuclear and cytoplasmic MxB protein forms hetero-oligomers and that the nuclear transport of the cytoplasmic MxB protein is facilitated by an interaction with the nuclear NLS-containing MxB protein via the C-terminal leucine zipper element. Cell fractionation studies also confirmed a significant presence of the cytoplasmic form of MxB protein in the nucleus. This observation demonstrates how the transport of a protein to the nucleus through a nuclear pore complex (NPC) occurs both by a specific NLS-requiring transport system and by a passive cotransport mechanism. Using confocal laser microscopy, we also show that the cytoplasmic MxA and the nuclear MxB proteins do not colocalize.

MATERIALS AND METHODS

Cells, Cultures, and Reagents—COS-1 (ATCC CRL 1650), human epithelioid carcinoma HEp-2 (ATCC CCL 25), and human glioblastoma T98G (ATCC CRL 1690) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin (0.6 μg/ml), streptomycin (60 μg/ml), glutamine (2 mM), HEPES buffer, pH 7.4 (20 mM), and 10% fetal calf serum (Integro, Zaandam, Netherlands). Primary human leukocytes were obtained from voluntary blood donors from the Finnish Red Cross Blood Transfusion Service, and peripheral blood mononuclear cells (PBMC) and macrophages were isolated as described previously (3). Human leukocyte IFN-α (6 × 10⁶ IU/ml) was kindly provided by Dr. Kari Cantell of our institute (24).

Antibodies—The details of the preparation and characterization of both polyclonal guinea pig and rabbit anti-human MxB and MxA antibodies (3) and monoclonal anti-influenza virus hemagglutinin 1-tag (HA1-tag) antibodies (25) have been described previously.

Plasmids and DNA Manipulations—MxB cDNA was modified as described elsewhere (3). To create a HA1-tagged (Tyr-Pro-Tyr-Asp-Val-Pro) transient expression plasmid, oligonucleotides with a new BamHI cloning site and BclI sites with cohesive ends (5'-stranded oligonucleotide, 5’GATCAACCATGTACCCCTACGACGTGCCCAGC-
Nuclear Transport of Human MxB Protein Complex

TACGCCGGATCCT and 3′-strand oligonucleotide, 5′GATCAGGATC-
GGCGTATGGCGCACGTTGAGGTAATTAGTTA(5′) were synthesized on an Applied Biosystems (Foster City, CA) model 394 DNA/RNA oligonucleotide synthesizer. The oligonucleotides were annealed, and the resulting double-stranded DNA fragment was subcloned into the BamHI site of the pBC12/CMV (7), a transient expression vector, to create a plasmid pBC12/CMV-HA1-tag. Gene constructs of wild-type MxB, MxB(25–715), lacking the 24 N-terminal amino acid-long NLS, MxB(43–715), and MxB(83–715), lacking both the NLS and the first and second proline-rich domains (PRD), respectively, have been described previously (3). Also the corresponding gene constructs, lacking a C-terminal leucine zipper domain, MxB(25–644) and MxB(83–644), were created by polymerase chain reaction (26) and subcloned into the newly created BamHI site of the pBC12/CMV-HA1-tag plasmid. The primers, which were used to modify the 5′ end of the MxB gene, were as described previously (3). The primer for the 3′ end was CTG AAT (GGA TCC) TCA TTA CTG GTT GGC GAC ACG-3′ (the BamHI site is in parenthesis, and the STOP codon is underlined). All the DNA manipulations were performed according to standard protocols (27), and the newly created gene constructs were partially sequenced. Various MxB gene constructs were also cloned into the BamHI site of pGEM-3Zf(+) (Promega) vector, and in vitro translation was carried out as previously described (28), using T7 Cap-Scribe and reticulocyte translation kits (Boehringer Mannheim GmbH, Mannheim, Germany).

**Transfections—**

Cos-1, Hep-2, and T98G cells were transfected with pBLCMV-MxB and various MxB deletion gene constructs, using the LipofectAMINE reagent (Life Technologies, Inc.) in accordance with the manufacturer instructions.

**Indirect Immunofluorescence Microscopy—**

Indirect immunofluorescence microscopy of MxA, MxB, and HA1-tagged MxB proteins were performed as described elsewhere (3) and photographed either on a Zeiss Axioshot photomicroscope or a Leica TCS NT confocal microscope.

**Subcellular Fractionation of PBMCs—**

IFN-α-induced (1000 IU/ml, 24 h) macrophages or T98G glioblastoma cells were washed twice with ice-cold phosphate-buffered saline, scraped from the dishes, and pelleted. All the cell manipulations were done on ice. The pelleted cells were washed twice with isoionic buffer A (10 mM HEPES, pH 7.4, 0.25 mM sucrose, 0.1 mM phenylmethylsulfonylfluoride (PMSF; Boehringer Mannheim GmbH), followed by disruption with a Dounce homogenizer (50 strokes) in 1 ml of buffer A, supplemented with 2 mM EDTA and 0.1 mM dithiothreitol. The nuclei were isolated by low speed centrifugation (50 strokes) and stained with anti-HA1 antibodies, the protein was found in a granular pattern in the cytoplasm (Fig. 2, d and f). If the cells were first treated with IFN-α, followed by transfection and fixing of these cells 12 and 48 h later, respectively, the HA1 epitope-tagged MxB protein was found in a granular pattern both in the nucleus and cytoplasm (Fig. 1, a and c). When the HA1-MxB(83–715) gene construct, lacking both the NLS and PRD, was expressed in uninduced cells and stained with anti-HA1 antibodies, the protein was found in a granular pattern in the cytoplasm (Fig. 2, e and h). The granular MxB-specific staining was found in the nucleus in only 2 to 3% of the cells (Fig. 3A), clearly suggesting that the leucine zipper element is mediating the oligomerization of the cytoplasmic and nuclear MxB proteins.

To consider the possibility that overexpression of various MxB gene constructs in COS-1 cells affected their transport into the nucleus, we carried out transfection experiments with IFN-α-induced MxB gene construct in cells that can naturally produce MxB protein in response to IFN-α stimulation. When Hep-2 and T98G cells were induced with IFN-α (1000 IU/ml, 24 h), MxB protein was found in a granular pattern both in the nucleus and cytoplasm (Fig. 2, a and c). Similarly, HA1-MxB(83–715) gene construct, lacking both the NLS and PRD, was expressed in uninduced cells and stained with anti-HA1 antibodies, the protein was found in a granular pattern in the cytoplasm (Fig. 1, a and c). If the cells were first treated with IFN-α, followed by transfection and fixing of these cells 12 and 48 h later, respectively, the HA1 epitope-tagged MxB protein was found in a granular pattern both in the nucleus and cytoplasm (Fig. 2, a and c). Nuclear staining of HA1-MxB(83–715) protein was found in 40 and 50% of T98G and Hep-2 cells, respectively (Fig. 3B), strongly suggesting that the natural, nuclear form of MxB protein can oligomerize with the HA1-MxB(83–715) protein and translocate it into the nucleus. If the experiment was done as above with a respective gene construct, also lacking the C-terminal leucine zipper element HA1-MxB(83–644), fluorescence was found diffusively in the cytoplasm (Fig. 2, d and h). The granular MxB-specific staining was found in the nucleus in only 3–5% of the cells (Fig. 3B).

**Equal Amounts of Nuclear and Cytoplasmic Forms of MxB Protein Exist in the Nucleus—**

To verify the cell biological observations of the cytoplasmic MxB protein being transported into the nucleus with the MxB protein (Fig. 1–3), we carried out cell fractionation experiments in cells or cell lines expressing human MxB as well as MxA proteins. First, we analyzed which of the two naturally expressed MxB-specific bands (78 and 76 kDa) in Western blotting (Ref. 3; Fig. 4A) corresponded to full-length or shorter MxB translation products. We used in vitro translation system with full-length and N-terminally truncated MxB gene constructs to verify their corresponding molecular masses. The full-length MxB gene construct was in vitro translated into two clearly detectable bands (78 and 76 kDa), corresponding exactly to the ones seen in primary leukocytes (PBMC, Fig. 4A). MxB gene construct, lacking the NLS namely MxB(25–715), was translated into a single band of 76 kDa, strongly suggesting that this band
corresponded to the in vivo translated cytoplasmic form of MxB protein. The in vitro translation products of the MxB(43–715) and MxB(83–715) gene constructs, initiating from the third and fourth methionine, gave rise to shorter MxB polypeptides (Fig. 4A).

To analyze the proportional amounts of the nuclear and cytoplasmic MxB proteins in the cell nucleus, IFN-α-induced macrophages and T98G glioblastoma cells were fractionated, and the nuclei and cytoplasmic extracts were analyzed in Western blotting. Based on densitometric scanning, the 78-kDa MxB protein comprised about 25% of the total MxB immunoreactivity in cells. In the nucleus, the proportional amounts of the 78- and 76-kDa forms of MxB protein were equal (50:50%) (Fig. 4B, lanes N), further supporting the view that also the cytoplasmic 76-kDa form of MxB protein is efficiently transported into the cell nucleus. Cytoplasmic MxA protein could not be found in the nucleus (Fig. 4B, lanes N; see also Fig. 5), indicating that the nuclei were virtually free of cytoplasmic contamination.

Since murine Mx1 protein is capable of forming oligomeric structures in vivo (21), we used chemical cross-linking of permethanilized IFN-α-induced T98G cells to analyze the potential oligomerization pattern of human MxB protein. Gel analysis revealed that dimers and trimers as well as other oligomeric forms of MxB protein were formed (Fig. 4C). This oligomerization pattern closely resembles the one seen for mouse and rat Mx1 proteins (21).

Nuclear MxB Protein Does Not Translocate the Cytoplasmic MxA Protein into the Nucleus—Since the cytoplasmic and nuclear forms of MxB protein can oligomerize via their leucine zipper elements, we addressed the question of whether the cytoplasmic MxA and cytoplasmic/nuclear MxB proteins would also form oligomers and colocalize in a natural situation, i.e. in IFN-α-treated primary human macrophages. The cells were treated with IFN-α (1000 IU/ml, 24 h), fixed, and double stained with rabbit anti-MxA and guinea pig anti-MxB antibodies, which showed very good specificity (3). Confocal laser microscopy revealed that MxA protein was found solely in the cell cytoplasm (Fig. 5A, a), whereas MxB protein was distributed evenly between the nucleus and cytoplasm (Fig. 5A, c). Colocalization analysis (Fig. 5A, b, B, and C) revealed that no MxA protein was found in the nucleus, clearly suggesting that
cytoplasmic MxA and nuclear MxB proteins do not form oligomers, and nuclear MxB protein cannot translocate cytoplasmic MxA protein into the nucleus. In the cytoplasm, there was some colocalization (Fig. 5A, b, yellow, and B) of MxA- and MxB-specific staining, but it is most likely due to localization of both proteins in similar network-like structures all over the cytoplasm. Quantitative analysis (Fig. 5, B and C) revealed that nuclear MxB protein accumulated underneath the nuclear membrane, and approximately 25% of the total MxB-specific staining was in the nucleus.

**DISCUSSION**

We have previously shown that humans also, not just rodents (7, 21–23), have an IFN-α/β-inducible nuclear form of Mx protein (3). Two forms of human MxB protein exist; an NLS-containing 78-kDa and a cytoplasmic 76-kDa form, the nuclear form comprising approximately 25% of total MxB immunoreactivity in primary human leukocytes. Deletion analyses revealed that the NLS of MxB protein is situated within the first 24 N-terminal amino acids of the protein (3). As detected by indirect immunofluorescence and immunoelectron microscopy, MxB protein was found in a granular pattern both in the cell cytoplasm and nucleus where it appeared to be localized in the nuclear matrix and associated to chromatin (3).

These findings encouraged us to examine the nuclear transport of the MxB protein in more detail and specifically ask the question of whether the cytoplasmic 76-kDa MxB protein, without an NLS, could also be transported into the nucleus. To be able to identify transfected forms of MxB protein from the endogenous ones, we created chimeric influenza virus hemagglutinin epitope-tagged MxB proteins. Based on transient transfection experiments in COS-1 cells, we could conclude that HA1-tagged proteins behaved as expected. The NLS-containing MxB protein was transported into the nucleus, whereas the MxB protein, lacking the NLS, remained in the cytoplasm (Fig. 1, a and e). These experiments also indicated that the full-length, nuclear 78-kDa MxB protein was capable of forming oligomers with the cytoplasmic 76-kDa or shorter forms of MxB protein and was able to cotransport these forms into the nucleus (Figs. 1 and 3). This suggests that MxB protein complexes, containing e.g. only one nuclear MxB protein form, would most likely be transported into the nucleus. When N-terminally truncated MxB proteins also lacked the C-terminal leucine zipper element (HA1-MxB(25–644) and HA1-MxB(83–644)), they remained in the cell cytoplasm (Figs. 1 and 3A), strongly suggesting that the C-terminal end is crucial in mediating the oligomerization of MxB proteins. An intact leucine zipper element is thus a prerequisite for the cytoplasmic form of MxB protein to be transported into the nucleus with the 78-kDa nuclear form. Conclusions based on the cotransfection experiments in COS-1 cells were fully supported by experi-

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**Fig. 2.** Subcellular localization of IFN-α-induced wild-type MxB protein and transiently transfected MxB gene constructs. HEp-2 and T98G cells were either induced with IFN-α (1000 IU/ml, 12 h) or left uninduced, followed by transfection with different MxB gene constructs as shown in the figure. The cells were fixed at 48 h after IFN-α-induction. Staining was performed with polyclonal guinea pig antibodies against MxB protein (a and e), followed by staining with FITC-labeled anti-guinea pig immunoglobulins or with monoclonal mouse antibodies against influenza virus HA1 epitope (b-d and f-h) followed by staining with FITC-labeled anti-mouse immunoglobulins. In the schematic representation below each panel (a-h), the functional domains of MxB, truncated and chimeric HA1-MxB protein, and MxB/HA1-MxB protein complexes are shown (the key of the functional domains is presented in Fig. 1). The corresponding gene constructs were either induced with IFN-α (a, c, d, e, g, and h; 1000 IU/ml) or transiently expressed in HEp-2 or T98G cells (b-d and f-h). IFN-α-induced wild-type MxB protein is found in a granular pattern both in the nucleus and cytoplasm in HEp-2 and T98G cells (a and e), whereas transiently expressed HA1-MxB(83–715) protein, lacking both the NLS and PRD, is found only in the cytoplasm (b and f). When the corresponding HA1-MxB(83–715) protein was transiently expressed in HEp-2 or T98G cells 12 h after IFN-α induction (1000 IU/ml) and stained 48 h post-induction with anti-HA1 antibodies, the granular fluorescence pattern was found both in the nucleus and cytoplasm as in panels a and e (c and g). When the cells were treated with IFN-α and stained as above but transfected with HA1-MxB(83–644) gene construct, lacking both the NLS, PRD, and leucine zipper element, the protein was found only in the cytoplasm (d and h). Bar, 10 μm.
ments carried out in HEp-2 and T98G cells in which the nuclear 78-kDa MxB protein was produced in response to IFN-α stimulation. In IFN-α-treated cells, the chimeric HA1-MxB(25–715) protein was found in the nucleus, indicating that endogenous, IFN-α-induced MxB protein cotransported the transfected cytoplasmic MxB protein into the nucleus. Similarly, if the C-terminal leucine zipper element was removed, no nuclear cotransport was taking place (Figs. 2 and 3B), further supporting the view that the leucine zipper element is crucial for oligomerization. However, in a minority of cells (2–5%), some fluorescence was found in the cell nucleus even if the chimeric protein lacked the C-terminal leucine zipper element. It is possible that some other parts of the MxB protein can mediate weak oligomerization, as has been suggested to be the case in human MxA and murine Mx1 proteins (30, 31). It has to be pointed out that, in using LipofectAMINE transfection system in COS-1 cell transfections, nearly all the transfected cells expressed both transfected gene products such as MxA and MxB proteins (from 80 to 95%, data not shown). Therefore, it is also likely that practically all the cells, transfected with different MxB gene constructs, expressed both forms of MxB protein.

However, formally, we could not control the cotransfection frequency since MxB-specific antibodies recognized both the tagged and untagged forms of MxB protein.

Cell fractionation studies further supported, at a more quantitative level, the idea that the cytoplasmic MxB protein was cotransported into the nucleus with the nuclear form of MxB protein (Fig. 4). In two studied cell types, namely primary human macrophages and T98G glioblastoma cells, at least 50% of the nuclear MxB immunoreactivity was of the shorter 76-kDa cytoplasmic form. These two types of cells were used since they express both MxA and MxB proteins, and thus MxA-specific immunostaining functioned as a control for the purity of the isolated nuclei. In the nuclei hardly any MxA protein was seen.

We have previously shown that human MxA protein as well as Mx proteins from other mammalian species could be chemically cross-linked to dimers, trimers, and larger oligomers both in vivo and in vitro (21). We and others have also demonstrated...
that the C-terminal parts of Mx protein, including the leucine zipper element, are responsible for the oligomerization of homotypic Mx proteins (21, 30). Our previous experiments with murine Mx1 protein (21) and the present experiments with human MxB protein indicate that the C-terminal leucine zipper element is an essential one in mediating the homo-oligomerization of Mx proteins. Cotransport experiments with transfected MxA and MxB proteins (results not shown) as well as the cell fractionation studies gave no indication that MxA protein would be transported into the nucleus with the nuclear form of MxB protein or that hetero-oligomers between MxA and MxB proteins would form. Quantitatively equal expression levels of MxA and MxB proteins (3) also allowed us to reliably analyze their possible colocalization in primary human leukocytes. Using confocal laser microscopy, only MxB protein was found in the nucleus, whereas both MxA and MxB proteins were found in a granular fashion in the cytoplasm with some colocalization (Fig. 5A, b). This minimal colocalization was possibly due to a high expression level of these proteins and their accumulation in the same or similar cytoplasmic network-like structures (Fig. 5). We believe that, even though MxA and MxB proteins have a C-terminal leucine zipper element, they are not able to form any hetero-oligomers with each other either in the nucleus or cytoplasm. Amino acid sequence analysis of the C-terminal ends of Mx proteins reveal that there are certain differences in the leucine repeats of MxB protein as compared with MxA protein. There are phenylalanine and isoleucine residues in MxB protein instead of leucines in the first and fourth positions of the leucine zipper element, respectively. It is possible that sequences apart from leucine zipper elements take part in the oligomerization, which only enables homo-oligomerization.

Our results clearly demonstrate that IFN-α-inducible human MxB proteins interact with each other via the C-terminal leucine zipper element, and oligomers with at least one NLS are transported into the cell nucleus, with the rest being destined to other structures in the cell cytoplasm. In the present study, we demonstrate a novel cotransport mechanism for the cytoplasmic human MxB protein. This includes a tight interaction between the leucine zipper elements of different molecules, followed by a “piggyback” ride of the cytoplasmic MxB protein with the nuclear form into the cell nucleus. This could enable a regulated transport mechanism of the cytoplasmic MxB protein into the nucleus depending on the tissue-specific relative expression level of the nuclear and cytoplasmic forms of MxB protein. However, a remaining question is the cellular function of MxB protein. Is it an antiviral protein against so far undetected viruses that have maturation steps both in the cell nucleus and cytoplasm, or has it some other profound effects on intracellular molecular traffic or cellular metabolism?

FIG. 5. Confocal images of indirect immunofluorescence staining for MxA and MxB proteins in primary human macrophages. Macrophages were treated with IFN-α (1000 IU/ml) for 24 h. A, a, staining for MxA protein with rabbit antisera, followed with FITC-labeled anti-rabbit immunoglobulins (green). c, staining for MxB protein with guinea pig antisera, followed with TRITC-labeled anti-guinea pig immunoglobulins (red). b, double staining and colocalization image (yellow) for MxA and MxB proteins. Focus was adjusted through the center of the nucleus. B, staining profiles of MxA (green) and MxB (red) proteins when the line was drawn through the cell as indicated in panel A. a, Cytoplasmic and nuclear areas are indicated, and the position of the nuclear membrane is pointed out by arrows. C, quantitation profiles of MxA (green) and MxB (red) proteins in the cell cytoplasm (98.7 ± 0.3% and 76.5 ± 1.8% of fluorescence signal, respectively) and nucleus (1.3 ± 0.3% and 23.5 ± 1.8% of fluorescence signal, respectively). Results were means of six different cells. Bar, 10 μm.
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Nuclear Transport of Human MxB Protein Complex 32359