Interferon-inducible Mouse Mx1 Protein That Confers Resistance to Influenza Virus Is GTPase*

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The murine Mx1 protein is an interferon-inducible nuclear protein and confers resistance to influenza virus infection even though the resistance mechanism is yet unclear. The Mx1 protein contains a tripartite GTP-binding domain consisting of GXXXGKS, DXXG, and T/NKXD motifs. In the GTPase gene superfamily such as p21** protein, signal-transducing G protein, and translation elongation factor, the GTPase activity plays a key role in each protein function. Here we show that GTPase activity is indeed associated with the intact Mx1 protein purified from *Escherichia coli* expressing Mx1 cDNA. Amino acid substitution within the GTP-binding motif led to significant reduction in the GTPase activity. Yeast vacuolar protein sorting (VPS1) protein and the rat microtubule-associated mecha-chemical enzyme dynamin were found to be homologous to Mx1 not only in the tripartite GTP-binding motif, but also in the amino-terminal region of ~300 amino acids in length. The function of Mx1 is discussed in comparison with these proteins.

The murine Mx1 protein is an interferon-inducible nuclear protein present in inbred mouse strains resistant to influenza infection, but not in strains sensitive to this virus (1). Mx1 cDNA was cloned from A2G mice resistant to influenza virus (2). Sequence analysis indicated that the Mx1 protein consists of 651 amino acids, >90% of which are charged, and contains at the carboxyl terminus a nuclear location signal that is essential for translocation of the Mx protein into nuclei (3). The Mx1 gene was mapped to mouse chromosome 16 and consists of 14 exons spread over >55 kilo base pairs of DNA (4). Two Mx± alleles were identified: one lacking three exons and the other containing a nonsense mutation that leaves a potential coding capacity for the amino-terminal half of the Mx1 protein (5). Expression of the Mx1 protein in influenza-sensitive cells and animals lacking this protein is sufficient to promote an antiviral state (2, 6). Moreover, microinjection of an anti-Mxl antibody into Mx± cells effectively neutralizes the antiviral state of interferon-treated cells (7). However, the molecular mechanism by which it confers this resistance is still far from being understood.

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Mx-like proteins and genes have been found in a number of vertebrates. For instance, two Mx-like proteins have been identified in human. Human Mx proteins are, however, present in cytoplasm. Moreover, human MxA produces a broader range of antiviral state than murine Mx1, preventing both orthomyxovirus and rhabdovirus infections (8), whereas the human MxB protein shows no detectable inhibition of viral infection. All the Mx proteins so far sequenced contain the tripartite GTP-binding consensus sequence typically found in GTP-binding proteins (8, 9). In this report, we show that the Mx1 protein is indeed a GTP-binding protein with GTPase activity by analyzing both the authentic Mx1 protein and a variant Mx1 protein that contains a point mutation in one of the GTP-binding domains. The function of the Mx1 protein is discussed in comparison with other GTPase proteins.

** MATERIALS AND METHODS

Antibodies against Mx1 Protein and Immunoblot Analysis—Two kinds of rabbit hyperimmune serum containing anti-Mx1 antibodies (anti-XN01 or anti-XC04) were prepared by immunizing animals with synthetic peptides corresponding to either the amino-terminal region (amino acids 79–89) or the carboxyl-terminal region (amino acids 617–631) of the Mx1 protein. For immunoblot analysis, proteins were separated by electrophoresis on 7.5% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) and electrophoretically transferred to a nitrocellulose filter (Schleicher & Schuell). The filter was exposed first to either the anti-XN01 or anti-XC04 antibodies and then to horseradish peroxidase-conjugated secondary antibodies against the rabbit a chain.

Purification of Mx1 Protein—*Escherichia coli* DH5 containing pTrpAl2Mx was grown in 20 liters of M9 minimal medium at 30°C (10). Cells were suspended in 80 ml of buffer A (20 mM HEPES/NaOH (pH 7.6 at 25°C), 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 200 mM KCl and disrupted with sonication. The extract was centrifuged at 12,000 rpm for 30 min and then at 30,000 rpm for 2.5 h. The supernatant was dialyzed against buffer B (20 mM HEPES/NaOH (pH 7.6 at 25°C), 0.1 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 200 mM KCl and subjected to chromatography on a phosphocellulose column (1.5 × 17 cm) previously equilibrated with buffer B containing 50 mM KCl. Proteins were fractionated with a 50–700 mM KCl linear gradient at a flow rate of 23 ml/h. The Mx1 protein fractions eluting around 270 mM KCl were pooled and dialyzed against buffer C (buffer A plus 10% glycerol) containing 50 mM KCl. The dialyzed sample was subjected to chromatography on a DAE-TOYOPEARL column (1.5 × 5.7 cm) previously equilibrated with buffer C containing 50 mM KCl. Proteins were fractionated with a 50–700 mM KCl linear gradient at a flow rate of 30 ml/h. The Mx1 protein fractions eluting around 255 mM KCl were pooled and fractionated by gel filtration through a Protein-Pak 300 column previously equilibrated with buffer containing 200 mM KCl. The pooled Mx fraction (5.9 mg) was

1 The abbreviations used are: SDS, sodium dodecyl sulfate; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography.
dialyzed against buffer C containing 50 mM KCl and subjected to SP-TOYOPEARL column chromatography. The column (0.7 x 6.5 cm) was eluted using a linear 50-700 mM KCl gradient at a flow rate of 16.8 ml/h. The Mx1 protein was eluted around 260 mM KCl.

**Assay of GTPase Activity**—The assay of GTPase activity was performed at 37 °C for 60 min in 25 μl of reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.1 mM dithiothreitol, 130 μM GTP, and 13 mM [α-35S]GTP (1 μCi, 3000 Ci/mmol). Products of the reaction were resolved by chromatography on polyethyleneimine-cellulose plates in 1.6 M LiCl.

**Construction and Purification of Mutant Mx1 Protein**—Expression vector pH3 was constructed by replacing the 37-base pair EcoRI-BamHI fragment of plasmid pH148 (11) with the 21-base pair EcoRI-BamHI fragment of M13mp18. For site-directed mutagenesis, the EcoRI-BamHI fragment of plasmid pHR148 (11) with the 21-base pair EcoRI-BamHI fragment of M13mp18. For site-directed mutagenesis, an oligonucleotide with the sequence (dAACAGATCTTCCG) was used as primer for DNA synthesis. In the resulting mutant Mx gene, Ser at position 50 of the Mx1 protein was substituted with Ile. Insertion of the mutagenized Mx1 cDNA into pH3 resulted in pS50I. The mutant Mx1 protein was purified as described above with slight modifications. In brief, the pellet after high-speed centrifugation of cell extracts was dissolved in buffer B containing 50 mM KCl and loaded onto a phosphocellulose column. After washing with a 5-column volume of buffer B containing 50 mM KCl, proteins were eluted with 700 mM KCl in buffer B. The eluted fraction was dialyzed against buffer C containing 50 mM KCl and directly applied to a Protein-Pak G-DEAE HPLC column (8.2 x 75 mm) equilibrated with the same buffer. The column was washed with 11.9 ml of buffer C, and then proteins were eluted with 39.9 ml of a 50-700 mM KCl linear gradient in buffer C at a flow rate of 0.7 ml/min. The Mx1 protein was eluted around 490 mM KCl.

**Binding of [α-32P]GTP to Mx Protein**—Samples were placed in a 96-well tissue culture plate on ice and irradiated with a UV source (Mineralight lamp UV254) at 18 watts at a distance of 5 cm for 30 min. Proteins were separated by electrophoresis in 7.5% polyacrylamide gel in the presence of 0.1% SDS. Labeled proteins were visualized by autoradiography of the gel. Radioactivity of the GTP binding to the Mx1 protein was measured using a Fuji Bio-Image analyzer.

**RESULTS**

**Purification of Mx1 Protein**—To prepare the intact murine Mx1 protein, plasmid pTrpΔ12Mx was constructed by placing the Mx1 cDNA clone (10) downstream of the E. coli trp promoter in the expression vector plasmid pH3 (11). A polypeptide with an apparent molecular mass of 72 kDa was produced in E. coli strain DH5 harboring the plasmid pTrpΔ12Mx but not the vector plasmid. For this purpose, the mutagenized Mx1 cDNA was inserted into the expression vector plasmid without Mx1 cDNA. In a typical experiment starting with 19 g of E. coli cells, 2.38 mg of pure Mx1 protein was recovered.

**Detection of GTPase Activity of Mx1 Protein**—The Mx1 protein contains a tripartite GTP-binding domain consisting of GXXXXGKS, DXXG, and T/NKXD motifs (8, 9). Using the purified Mx1 protein, we examined whether GTPase activity is associated with Mx1 or not. After incubation with [α-32P]GTP, reaction products were analyzed by thin-layer chromatography on polyethyleneimine-cellulose using either 1.0 or 1.6 M LiCl. As illustrated in Fig. 2C, [α-32P]GTP was converted to [α-32P]GDP, and this GTPase activity co-chromatographed with the Mx1 protein. Under the same reaction conditions except that [α-32P]GTP was replaced with one of the other 32P-labeled nucleoside triphosphates, the hydrolysis of ATP was ~37% of that of GTP hydrolysis, but that of CTP and UTP was below the detection level (data not shown).

**Detection of GTPase Activity of Mx1 Protein**—The Mx1 protein thus expressed in E. coli was purified to near-homogeneity through four steps, i.e., chromatography on phosphocellulose and DEAE-TOYOPEARL, gel filtration through Protein-Pak 300, and chromatography on SP-TOYOPEARL. Fig 2A shows a Coomassie Blue-stained SDS-polyacrylamide gel of fractions separated by chromatography on SP-TOYOPEARL. The band of 72-kDa protein formed a single and major peak at 260 mM KCl. Immunoblot analysis using polyclonal anti-Mx1 antibodies confirmed that this major 72-kDa protein was the Mx1 protein (Fig. 2B). Other minor cross-reactive bands may be related to the Mx1 protein because these proteins were copurified along the Mx1 protein up to the final step and because such proteins could not be identified in corresponding fractions from a lysate of E. coli containing the expression vector plasmid without Mx1 cDNA.

**Fig. 1. Characterization of rabbit anti-Mx1 antisera raised against synthetic peptides.** Rabbit anti-Mx1 antisera was raised against synthetic peptides. Primary cultures of A2G mouse embryos were grown for 18 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 1000 units of interferon-α/β per ml. An aliquot of each cell lysate (85 μg of protein) was subjected to immunoblot analysis using either anti-XNO1 (lanes 1 and 2) or anti-XCO4 (lanes 3 and 4) antibodies. The migration positions of marker proteins (myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and trypsin inhibitor (21.5 kDa)) are shown on the left.
recovered as precipitates by centrifugation for 2.5 h at 30,000 rpm. The pellet was dissolved and absorbed onto phosphocellulose, and a one-step eluate at 0.7 M KCl was fractionated on a polyethyleneimine-cellulose column. The first lane contained no fraction. The positions of AMP, CMP, GMP, UMP, GDP, and GTP were identified using unlabeled reference nucleotides.

Fig. 2. Cochromatography of Mx1 protein and GTPase activity. A, purification of the Mx1 protein. The distribution of proteins in aliquots (5 µl each) of the indicated fractions from SP-TOYOPEARL column chromatography was analyzed by electrophoresis on 7.5% polyacrylamide gel in the presence of SDS. The gel was stained with Coomassie Brilliant Blue. The migration positions of marker proteins are shown on the left. B, immunoblot analysis of the Mx1 protein. An aliquot (5 µl) of each fraction as shown in A was analyzed by Western blotting using the anti-X04 antibody specific for the Mxl protein. Lane L, 25 µg of a cell extract from A2G mouse liver after injection of poly(I)·poly(C). The migration positions of the marker proteins are shown on the left, C, assay of the GTPase activity. The GTPase assay was performed at 37 °C for 60 min as described under "Materials and Methods" using aliquots (5 µl each) of the indicated SP-TOYOPEARL column chromatography fractions. Products of the reaction were resolved by chromatography on a polyethyleneimine-cellulose plate in 1.6 M LiCl. The first lane contained no fraction. The positions of AMP, CMP, GMP, UMP, GDP, and GTP were identified using unlabeled reference nucleotides.

mutant Mx1 protein was only one-fourth the level of the wild-type Mxl protein, whereas virtually no GTPase activity was detected for the same volume of the equivalent column fraction from cells without Mx1 cDNA. These observations indicate that GTPase is an intrinsic activity associated with the Mxl protein and the GXXXXGKS motif plays a role in this GTPase activity. The calculated turnover number for the wild-type Mx protein is 5.8 mol of GTP hydrolyzed per min/mol of protein at 37 °C.

Detection of GTP Binding Activity of Mx1 Protein—To demonstrate direct interaction between the Mxl protein and GTP, we attempted UV photoaffinity labeling (14). When the wild-type Mxl protein was exposed to UV light in the presence of [α-32P]GTP, a single band of labeled protein was detected after gel electrophoresis (Fig. 4). No proteins were labeled without UV irradiation (data not shown). Likewise, no band appeared when several unrelated proteins (β-galactosidase, bovine serum albumin, and ovalbumin) were tested by this treatment. The Mxl protein cross-linked with GTP migrated a little slower than the untreated Mxl protein. This band...
was, however, not detected for the fraction derived from an extract of cells containing hHR3. These observations indicate that the Mx1 protein physically interacts with GTP. The level of GTP cross-linked to the wild-type Mx1 protein was 4.2-fold higher than that cross-linked to the mutant Mx1 protein. This ratio of GTP binding is in good agreement with the ratio of GTPase activity. Thus, we concluded that the amino acid change from Ser to Ile at position 50 of the Mx1 protein resulted in a decrease in the GTPase activity due to reduction in GTP binding affinity.

**DISCUSSION**

In the family of GTP-binding proteins including signal transducing G protein and elongation and initiation factors involved in protein synthesis, the GTPase activity plays a regulatory role in each protein function. More recently, low molecular mass GTP-binding proteins related to the ras proto-oncogene have been implicated in playing a role(s) in regulating membrane vesicle budding and fusion involved in transport from the endoplasmic reticulum through the Golgi complex as well as in processes of endocytosis and exocytosis. ras-related GTP-binding proteins also appear to be involved in cytoskeletal polymerization. Furthermore, secretory protein transport across the endoplasmic reticulum is mediated by the receptor of the signal recognition particle, which has been shown to be a GTP-binding protein. When compared to typical enzymatic reactions, the rates at which these GTP-binding proteins convert GTP to GDP are relatively slow, ranging from 12 to 250 mmol/min/mol of protein at 37°C (15). The rate of GTP hydrolysis by the Mx protein is also slow (~6 mol/min/mol of protein). Thus, the Mx1 protein may act as a molecular switch like these GTPase family proteins.

The GTPase family proteins are interconvertible among three conformational states, i.e., GDP-bound, empty, and GTP-bound, by a cyclic reaction of binding and hydrolysis of GTP (16). For many GTPases, the cycle of GTP binding and hydrolysis takes place involving two regulatory components: a guanine nucleotide release protein and a GTPase-activating protein. Similar regulatory proteins may therefore be involved in the control of antiviral activity of the Mx1 protein. Alternatively, if the Mx1 protein is categorized into another GTPase family such as tubulin, the Mx1 protein itself could be reversibly assembled by the medium of GTP hydrolysis. The assembled form of the Mx1 protein may be active in the establishment of the antiviral state.

The mouse Mx1 protein is located in the nucleus (2), whereas the human MxA protein is a cytoplasmic protein. The mouse Mx1 protein confers selective resistance to influenza virus, whereas the human MxA protein can inhibit both influenza virus and vesicular stomatitis virus. Nevertheless, the amino-terminal regions of the Mx protein family, including murine (Mx1 and Mx2), rat (Mx1 to Mx3) (17), human (MxA and MxB), and fish Mx proteins, show a high degree of sequence conservation (18). The conserved regions contain a tripartite GTP-binding domain, suggesting that all the Mx family proteins have GTPase activity and that this GTPase activity is essential for the antiviral activity.

Moreover, the Mx protein appears to have an important cellular function(s) because Mx homolog proteins have been identified in wide varieties of animal species including monkey, pig, cattle, sheep, horse, dog, cat, and rabbit (19) and because the Mx homolog protein has been shown to be essential for the viability of yeast. Along this line, it is worthwhile to note that the murine Mx1 protein is homologous to not only the Mx family proteins, but also to two other cellular proteins. One is the product of yeast vacuolar protein sorting (VPS1) protein (20). Yeast cells contain a prominent vacuole that is considered to be equivalent to mammalian lysosome because of its low pH. Vacuolar protein sorting (VPS) encodes a protein required for sorting of newly synthesized vacuolar proteins from secretory proteins during their transport through the yeast secretory pathway. The other is the rat microtubule-associated mechochemical enzyme dynamin (21). Microtubules are involved in various steps of intracellular motility. Cytoplasmic microtubules are composed of tubulin and a diverse set of microtubule-associated proteins. Dynamin is one of the microtubule-associated proteins, but is distinct from dynein and kinesin. Dynamin forms periodic cross-bridges between microtubules and converts them into highly organized bundies. The amino-terminal region of ~300 amino acids is highly conserved among Mx1, vacuolar protein sorting gene 1, and dynamin (21). The tripartite GTP-binding domain exists in this conserved region, suggesting that both vacuolar protein sorting (VPS1) protein and dynamin are also GTPases. In fact, a low level of GTPase activity, i.e., ~1 mol/min/mol of protein (cited in Ref. 22), was detected for dynamin in addition to A or B activity. This GTPase activity is markedly stimulated by microtubules. Along this line, it would be worthwhile to test possible effects of microtubules on the GTPase activity of the Mx1 protein.

The Mx protein may therefore be involved in either protein sorting or intracellular motility. In the case of the human MxA protein, it is a cytoplasmic protein and inhibits growth of both influenza virus and vesicular stomatitis virus. Furthermore, it is noteworthy that vesicular stomatitis virus, Newcastle disease virus, and Sendai virus require cytoplasmic microtubule-associated proteins, cytoskeleton, and tubulin, respectively, for transcription and/or replication (23, 24). Likewise, the murine Mx1 protein may interact with a nuclear structure such as the nuclear matrix. If so, it is possible that the growth of influenza virus may depend on the nuclear structure.

In the case of p21"", the level of GTPase activity is closely related to its transformation activity (24). To understand whether the GTPase activity of the Mx1 protein is indeed important for its antiviral activity, a systematic comparison is being made between the degree of influenza virus resistance and the level of GTPase activity for mutant Mx1 proteins using both cultured cell and transgenic animal systems. As a further extension of this line of study, antiviral animals could be established by introducing mutant Mx genes that carry either accelerated or decelerated levels of the GTPase activity.

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