Role of GTPase activity of murine Mx1 protein in nuclear localization and anti-influenza virus activity

Tetsuya Toyoda, Yukiyasu Asano† and Akira Ishihama*

Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka, 411 Japan

Murine Mx1 protein is an interferon-inducible GTPase which localizes in nuclei and inhibits influenza virus infection. Wild-type Mx1 and two mutant Mx1 proteins, each carrying a single mutation either in the GTP-binding motif (S50I) or in the self-assembly motif (C71S), were expressed in MDCK cells. Wild-type Mx1 localized in nuclei, forming small granules with minute dots, and inhibited influenza virus growth. Mutant S50I, which had no GTP-binding or GTPase activities, formed linear structures in nuclei and lacked anti-viral activity, while C71S appeared diffuse in nuclei as minute dots without granules, but retained the inhibitory activity against influenza virus growth. A correlation existed between GTPase activity, intranuclear distribution and antiviral activity. We concluded that GTPase activity is essential for expression of the biological activity of Mx1 protein.

Murine Mx1 protein is an interferon-inducible nuclear protein present in the A2G mouse strain, which is resistant to influenza virus infection. Expression of the Mx1 protein in cells and transgenic animals permissive for influenza virus infection induces an anti-influenza virus state in the absence of interferon, indicating that Mx1 itself inhibits influenza virus growth (Stacheli et al., 1986; Kolb et al., 1992). Biochemical analysis indicated that Mx1 is a GTP-binding protein with GTPase activity (Nakayama et al., 1991, 1992; Melén et al., 1994) and that Mx1 forms self-assemblies (Nakayama et al., 1993). The functional domains of Mx1 involved in GTP-binding, GTP hydrolysis, self-assembly, di- and trimerization, and nuclear localization have been mapped (Noteborn et al., 1987; Nakayama et al., 1991, 1992, 1993; Melén et al., 1992, 1994). Mutant analysis indicated that nuclear localization, GTP binding and GTPase activities, and dimer or trimer formation are essential for its anti-influenza virus activity (Zürcher et al., 1992; Garber et al., 1993; Pitossi et al., 1993). For detailed analysis of the structure-function relationship of Mx1 protein, we created two Mx1 mutants, S50I and C71S, carrying a single amino acid substitution in the GTPase or self-assembly domain, respectively, expressed them in eukaryotic cells and examined their biological activities.

The fragments containing Mx1 and its mutant cDNA, S50I, were cloned from pTrpA12Mx and pS50I (Nakayama et al., 1991), respectively, into the expression vector pCB6 (obtained from Dr M. Roth, University of Texas, Tex., USA, with permission from Dr M. Stinski, University of Iowa, Iowa, USA) between BglII and HindIII to create pCBMx1 and pCBS50I, respectively. In order to substitute the cysteine at position 71 of Mx1 with serine, two DNA fragments were produced by PCR using pTrpA12Mx as a template and two pairs of primers: YA1, d(GGGAATTACGATGGATTCTGTAGAATAATCTGTGC) and YA2, d(AGGAGATCTGGTGACAATACCACCTGC); YA3, d(ACCAGATCTCCTCTGGTGTGACTCAACCACCACCACCTGC); YA3, d(ACCAGATCTCCTCTGGTGTGACTCAACCACCACCACCTGC) and YA4, d(AGCTCCCTCAGTCGACTCTCTCTC). The resulting two fragments were inserted together into pTrpA12Mx between the EcoRI–SalI sites by a three fragment ligation method to produce pC71S, which had a single substitution of cysteine by serine at position 71. Finally, an EcoRI–HindIII fragment of pC71S was recloned into pCMV5 (a gift of Dr M. Stinski) to generate pCMVC71S.

The wild-type and mutant Mx1 (S50I and C71S) cDNAs were transfected into MDCK cells and three independent clones stably expressing the Mx1 protein (CK/pCB6, CK/Mx1, CK/S50I and CK/C71S) were established after G418 (Gibco BRL) selection. Intra-cellular localization of three types of Mx1 protein was studied by indirect immunofluorescence microscopy using an anti-Mx1 antibody, XC04, which was raised in rabbits against a synthetic peptide with the Mx1 C-terminal sequence comprising amino acids 617–631.

* Author for correspondence. Fax +81 559 81 6746. e-mail aishihama@lab.nig.ac.jp

† On leave of absence from Rational Drug Design Laboratories, Fukushima, Fukushima 960-12, Japan.
Fig. 1. (a) Molecular map and intranuclear localization of Mx1 and its mutant proteins S50I and C71S. The filled boxes in the map (amino acids 43–50, 144–147 and 213–216) represent the GTP-binding domain; the striped box (amino acids 50–99) represents the self-assembly domain; the checked box (amino acids 602–631) represents the nuclear localizing signal; the grey and checked boxes (amino acids 565–631) represent the leucine zipper motif. Indirect immunofluorescent micrographs using anti-Mx1 antibody, XC04 (1:2000), followed by treatment with rhodamine B-conjugated anti-rabbit IgG (1:200; TAGO), are also shown. A2G, A2G embryonic cells treated with interferon; Mx1, CK/Mx1-1 cells; S50I, CK/S50I-1 cells; C71S, CK/C71S-1 cells. Scale bar represents 3.6 μm. (b) Western blot analysis of Mx1-expressing cell lysates. Cell lysates containing 50 μg of total cellular proteins were fractionated by electrophoresis on SDS–polyacrylamide (5–15%) gels and electroblotted onto PVDF membranes. Western blot analysis was carried out using anti-Mx1 antibody, XC04 (1:2000) and alkaline phosphatase-conjugated anti-rabbit IgG (1:15000; Promega), followed by development with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The position of Mx1 and its mutant proteins is indicated by an arrowhead. The migration positions of the molecular mass standards are indicated on the left. The cell clones used were: lane 1, A2GEC treated with interferon; lane 2, MDCK cells; lane 3, CK/pCB6-1; lane 4, CK/Mx1-1; lane 5, CK/S50I-1; lane 6, CK/C71S-1.

(Nakayama et al., 1991). Fig. 1(a) shows the intranuclear distribution of wild-type and mutant Mx1 proteins in MDCK cells. All of the Mx1 proteins localized in the nucleoplasm, but not in the nucleoli. Wild-type Mx1 formed small granules and minute dots. This pattern is similar to that observed for the authentic mouse Mx1 protein expressed in A2G embryonic cells, A2GEC, after mouse α- and β-interferon induction (2000 U/ml; Fig. 1(a)). S50I mutant Mx1 formed linear or tubular structures with faint minute dots. In contrast, C71S did not form any large granules, but formed fine minute dots. When these plasmids were transfected into COS-7 cells, the intranuclear localization and the shape of Mx1 aggregates were essentially identical to those in stably expressing MDCK cells, except that in some S50I-expressing COS-7 cells, linear elements were observed in both nuclei and cytoplasm (data not shown).

The expression of the Mx1 proteins in MDCK cells was also confirmed by Western blot using the anti-Mx1 antibody, XC04 (Fig. 1(b)). The level of expression of Mx1 protein was then measured by quantitative Western blot analysis using the Escherichia coli-expressed Mx1 protein as a standard (Table 1). After interferon induction, embryonic cells of A2G mice expressed 85 ng of Mx1 protein per 1 mg of total cellular protein. In the high-level expressing clones of the wild-type and S50I Mx1-transfected cell lines, the expression levels were equivalent to those in interferon-induced A2G cells. The level of C71S expression was, however, less than those of wild-type and S50I Mx1.

Next we examined the inhibition of influenza virus growth by these mutant Mx1 proteins using plaque assays. First, we isolated independent clones for each of the three wild-type Mx1- and mutant Mx1-expressing MDCK cell lines, and infected them with influenza virus A/P8/34 at a m.o.i. of 0.1 p.f.u./cell. The supernatant was harvested at 12 h after infection and the virus yield was determined by plaque assay on MDCK monolayers. Table 1 shows the virus yield normalized with respect to three independent clones of pCB6-transfected MDCK cells. Wild-type Mx1-expressing cell lines (CK/Mx1-1, CK/Mx1-2 and CK/Mx1-3) exhibited 1000–10000-fold reductions in virus yield, and C71S mutant-expressing cells (CK/C71S-1, CK/C71S-2 and CK/C71S-3) ex-
Alternatively, the cysteine at position 71 may play a role was, however, about 10% of the level in A2G cells in inhibition of virus growth. The expression level of C71S bound [\(\sim\text{T}-32\text{P}\)]GTP as well as wild-type Mx1 (data not shown). C71S may also have GTPase activity as high as wild-type Mx1, since the GTP-binding activity of Mx1 parallels GTPase activity (Nakayama et al., 1991; Pitossi et al., 1993).

The correlation between the GTPase activity and the intranuclear distribution suggests that minute dots in nuclei represent an active state of the Mx1 protein while small granular forms represent inactive molecules. S50I is a loss-of-function mutant like most of the Mx1 mutants so far isolated. A single amino acid mutation in the leucine zipper motif also leads to loss of antiviral activity (Garber et al., 1993). Except for C71S, only one mutant of Mx1 which retained its antiviral function has been reported (C47; Pitossi et al., 1993).

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Table 1. Mx1 levels and influenza virus growth inhibition in Mx1-expressing cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mx1 level* (ng/mg total protein)</th>
<th>Plaque formation (%)†</th>
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<tbody>
<tr>
<td>MDCK</td>
<td>ND</td>
<td>130</td>
</tr>
<tr>
<td>CK/pCB6</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>CK/Mx1-1</td>
<td>44±7.0</td>
<td>001</td>
</tr>
<tr>
<td>CK/Mx1-2</td>
<td>88±14</td>
<td>003</td>
</tr>
<tr>
<td>CK/Mx1-3</td>
<td>7±3.17</td>
<td>0-1</td>
</tr>
<tr>
<td>CK/S50I-1</td>
<td>52±7.6</td>
<td>48</td>
</tr>
<tr>
<td>CK/S50I-2</td>
<td>70±8.4</td>
<td>53</td>
</tr>
<tr>
<td>CK/S50I-3</td>
<td>3±3.021</td>
<td>57</td>
</tr>
<tr>
<td>CK/C71S-1</td>
<td>1±1.8</td>
<td>5-4</td>
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<tr>
<td>CK/C71S-2</td>
<td>8±1.4</td>
<td>7-5</td>
</tr>
<tr>
<td>CK/18G-3</td>
<td>7±1.4</td>
<td>5-4</td>
</tr>
<tr>
<td>A2G/EC</td>
<td>85±13</td>
<td>ND</td>
</tr>
</tbody>
</table>

* The levels of Mx1 proteins were measured by quantitative Western blotting and are calculated from three independent experiments (average ± SD; \(r^2 > 0.96\)). ND, Not determined.
† The values represent the average of three independent experiments.
‡ 100% value represents the average of plaque numbers on three independent clones of CK/pCB6 cell lines.

References


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