Self-assembly of Human MxA GTPase into Highly Ordered Dynamin-like Oligomers*

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Human MxA protein is a member of the interferon-induced Mx protein family and an important component of the innate host defense against RNA viruses. The Mx family belongs to a superfamily of large GTPases that also includes the dynamins and the interferon-regulated guanylate-binding proteins. A common feature of these large GTPases is their ability to form high molecular weight oligomers. Here we determined the capacity of MxA to self-assemble into homo-oligomers in vitro. We show that recombinant MxA protein assembles into long filamentous structures with a diameter of about 20 nm at physiological salt concentration as demonstrated by sedimentation assays and electron microscopy. In the presence of guanosine nucleotides the filaments rearranged into rings and more compact helical arrays. Our data indicate that binding and hydrolysis of GTP induce conformational changes in MxA that may be essential for viral target recognition and antiviral activity.

Human MxA protein belongs to a family of highly conserved GTPases that have been discovered because some Mx family members have antiviral activity against RNA viruses (1). The Mx GTPase family belongs to a superfamily of large GTPases that includes, among others, the dynamins and the guanylate-binding proteins (2). Mx proteins are abundantly expressed in interferon-treated cells (3) and play a crucial role in the early antiviral defense against certain RNA viruses as clearly demonstrated in studies with Mx transgenic mice (4–6). Other members of this superfamily are involved in fundamental cellular processes such as endocytosis (7), intracellular vesicle transport (8), organelle maturation and organelle division (9–11), and cell wall formation in plants (12).

Human MxA is a 76-kDa protein with low affinity for GTP and a high GTP turnover rate (13, 14). Interestingly the GTPase activity of MxA appears to be independent of cofactors such as GTPase-activating proteins or nucleotide exchange factors (2). This intrinsic GTPase activity is a characteristic feature of all members of the superfamily of dynamin-like large GTPases (15, 16). Furthermore, a feature common to most family members is their localization to intracellular membranes and their involvement in intracellular membrane fission processes. In contrast, the Mx proteins seem not to be membrane-associated and are not involved in membrane fission but are involved in the early interferon-induced antiviral response (17).

The domain structure of large GTPases is now well established. All family members have a highly conserved tripartite GTP binding motif within their N-terminal G domains (15). GTP binding and/or hydrolysis are required for function (18). This has also been demonstrated for the antiviral activity of Mx proteins (19, 20). The full-length crystal structure of the human guanylate-binding protein (21) and the crystal structure of the G domain of dynamin A of Dictyostelium discoideum (22) have recently been solved. They represent the first known structures in the superfamily of large GTPases. The globular G domains of both proteins resemble the G domain of Ras-like small GTPases but exhibit also some specific differences. It is likely that the G domains of Mx proteins have similar architectural features.

The C-terminal parts of the molecules are less well conserved among the various members of the dynamin superfamily and serve specific functions. They control self-assembly and association with effector molecules (23, 24). Three different regions have been described to be involved in the self-assembly process of dynamin-like GTPases (24–26): (i) an N-terminal “self-assembly” sequence that is conserved in all members of the large GTPase superfamily (27–29), (ii) a “middle” or “central interactive domain” that mediates oligomerization via interaction with the C-terminal part of the molecule (20, 28, 30, 31), and (iii) an assembly domain at the extreme C terminus that is rich in α helices and, in Mx proteins, contains a leucine zipper motif (32). This region interacts with the “middle domain” and the N-terminal self-assembly sequence (28, 30, 31, 33), which results in an increased GTPase activity indicating that this C-terminal region acts as a “GTPase effector domain” (33–35). Dynamin has additional C-terminal domains that are important for its function. The “pleckstrin homology region” is involved in the association of dynamin with phospholipids, and the “proline-rich domain” mediates binding to partner molecules with a Src homology 3 domain (25). These domains are absent in Mx protein family members that do not seem to associate with membranes.

Homo-oligomerization into ring-like and helical structures is essential for the biological function of dynamin-like proteins (18, 29, 36). Self-assembly of dynamin, for example, stimulates its GTPase activity (35, 37) and is crucial for dynamin-mediated endocytotic processes (35, 38, 39). It has been shown that the hydrolysis of GTP to GDP causes a conformational change that alters the structure of dynamin aggregates (40). The conformational change presumably generates the mechanochanical force necessary for the vesiculation of lipid structures (18, 26). Mx proteins also form homo-oligomers of high molecular weight.
weight (14, 32) and self-assemble into “horseshoe”-like structures (27). We have recently demonstrated that human MxA binds to viral nucleocapsids in vitro (41). This interaction requires the presence of GTPyS, a nonhydrolyzable GTP analog, as a stabilizing factor. Most likely GTP-binding leads to a conformational change that favors tight binding to the viral target structures (24).

To understand the mechanism of MxA action, we studied the capacity of different salt concentrations and different types of guanosine nucleotides to change MxA conformation and self-assembly. We show that recombinant MxA self-assembles into ordered ring- and spiral-like structures. The shape of these structures changed depending on the type of guanosine nucleotide bound. These data suggest that MxA displays different conformations in the course of its GTPase cycle.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant MxA—**Histidine-tagged MxA and MxA(T103A) were produced in *Escherichia coli* and isolated using Ni²⁺ chelate chromatography as described previously (19, 20). The proteins were further purified by Mono Q ion exchange chromatography in 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 10% glycerol, 300 mM NaCl (24). The recombinant histidine-tagged proteins had a concentration of about 3–4 mg/ml and were used for analysis immediately without freezing in all experiments described.

**Pellet Fraction Assay—**Recombinant MxA was diluted to a concentration of 0.5 mg/ml in HCB300 (20 mM Hepes, pH 7.5, 1 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol containing 300 mM NaCl) and dialyzed against HCB overnight at 4°C. When indicated, guanosine nucleotides (100 μM) or GDP/AlF₄⁻ (100 μM GDP combined with 5 mM NaF and 500 μM AlCl₃) were added to the dialysis buffer or were added directly into the protein solution prior to incubation at 37°C for 10 min. Samples (300 μl) were centrifuged at 100,000 × g for 15 min at 4°C (50,000 rpm in a TLA120 rotor, Beckman Instruments). Pellets were resuspended in 100 μl of RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS). Supernatants (S) and pellets (P) were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Molecular mass markers (in kDa) are indicated on the right bar, electron micrographs of negatively stained MxA oligomers after dialysis overnight at 4°C in HCB with different salt concentrations as indicated. Final protein concentrations were 0.1 mg/ml. Scale bar, 150 nm.

**RESULTS**

**MxA Forms Filamentous Structures under Low Salt Conditions—**To study the conformational changes of MxA protein resulting in oligomer formation, we first determined the salt dependence of protein self-assembly. The oligomerization state of MxA was determined by a sedimentation assay as described by Hinshaw and Schmid (40) and by electron microscopy. For this purpose, recombinant MxA isolated from *E. coli* in high salt buffer was dialyzed against various salt concentrations at 4°C. MxA protein was predominantly found in the supernatant after ultracentrifugation in the presence of 300 mM NaCl, whereas only a small part of the protein sedimented into the pellet fraction (Fig. 1A). This suggests that MxA existed predominantly in a low oligomeric state at high salt concentration. Electron micrographs of the protein solution before centrifugation revealed granular as well as some spherical or short fibrillar structures (Fig. 1B, 300 mM). After lowering the salt concentration in the dialysis buffer, MxA appeared in the pellet fraction following ultracentrifugation (Fig. 1A). Electron micrographs confirmed this change in self-assembly of MxA under low salt condition yielding long filamentous structures with a diameter of about 20 nm (Fig. 1B, 50 and 150 mM NaCl). Previous gel filtration studies are in agreement with the polymeric state of MxA under low salt conditions. It was estimated that the molecular weight of these MxA polymers was ~2 MDa, suggesting that they consist of about 30 monomers (14, 24).

**Binding of GDP/AlF₄⁻ Results in Strong Oligomerization under High Salt Condition—**To investigate the influence of nucleotide binding on MxA self-assembly by the sedimentation assay, we used high salt buffer (300 mM NaCl) conditions and various GTP analogs. In a first set of experiments, MxA was dialyzed against HCB300 (see “Experimental Procedures”) with different nucleotides at 4°C overnight. The nonhydrolyzable nucleotide analogs GDP/PS and GTPyS that mimic binding of GDP or GTP to MxA showed no effect on the sedimentation behavior (Fig. 2A). However, when MxA was incubated with GDP in the presence AlF₄⁻, the majority of MxA was found in the pellet fraction. It has been shown that AlF₄⁻ binds preferentially to the GDP-loaded form of GTP-binding proteins thereby representing the conformation of the transition state during GTP hydrolysis (42–44). Similar results were obtained when MxA was first dialyzed against HCB300 before adding the various nucleotides and following a 10-min incubation at 37°C (Fig. 2C and data not shown). To confirm the specificity of the GDP/AlF₄⁻–dependent effect, we analyzed the behavior of MxA(T103). This mutant protein has a threonine to alanine exchange at position 103 in the GTP-binding domain that significantly lowers the GTP binding capacity of MxA (20). When dialyzed against various salt concentrations, MxA(T103) behaved like the wild-type protein in the sedimentation assay. Accordingly the mutant protein was found in the pellet fraction.
in the presence of 150 mM NaCl but in the supernatant when 300 mM NaCl was used (Fig. 2B). Incubation of MxA(T103A) with GDP/AlF₄⁻ at 37 °C for 10 min in high salt buffer had no effect on its oligomerization state, whereas the wild-type protein sedimented after binding of GDP/AlF₄⁻ as expected (Fig. 2C). These data indicate that oligomerization of MxA in the presence of GDP/AlF₄⁻ requires a functional nucleotide-binding domain.

**MxA Forms Ring- and Spiral-like Structures under Low Salt Conditions**—The effect of various nucleotides on MxA self-assembly was studied by electron microscopy. MxA was diluted in low salt buffer (50 mM NaCl) on ice, resulting in the formation of the characteristic filamentous structures (Fig. 3A, 50 mM NaCl (4 °C)). When this protein solution was subsequently incubated at 37 °C for 10 min, the filaments disassembled into shorter, more condensed aggregates (Fig. 3A, 50 mM NaCl (37 °C)). Using these experimental conditions, MxA was mixed with different types of guanosine nucleotides before incubation at 37 °C for 10 min. Then the protein solutions were analyzed by ultracentrifugation. WT, wild type.

**DISCUSSION**

We have studied the self-assembly of MxA into high molecular weight structures. Oligomerization of MxA was influenced by salt concentration, temperature, and presence of guanosine nucleotides. Under low salt conditions at 4 °C, MxA formed long fibrillar structures that disintegrated into smaller fragments after incubation at 37 °C. Binding of GDP/βS under low salt conditions at 37 °C induced the formation of evenly shaped rings that condensed to spirals or stacks of rings after incubation with GTPγS. A complex of MxA with GDP/AlF₄⁻ yielded larger oligomeric granula and may thus mimic the conformation of MxA in a transition state during GTP hydrolysis.

Interestingly MxA self-assembled into large filamentous structures with an approximate diameter of about 20 nm when exposed to salt concentrations below 300 mM. This was remarkable because similarly large polymers are formed by dynamin only at salt concentrations below 50 mM (40). At higher salt concentrations, dynamin forms tetramers (34). In contrast, MxA forms low molecular weight oligomers such as dimers and trimers only when unphysiological buffer conditions (1 M NaCl, 50% ethylene glycol) are used (32). These findings indicate that MxA differs markedly from dynamin in its propensity to oligomerize. Therefore, MxA is likely to form large oligomeric structures even at physiological salt concentrations in living cells. MxA has indeed been observed to aggregate into punctate granula in the cytoplasm of interferon-treated human cells or in cells transfected with cDNA expression constructs coding for MxA (45, 46).

MxA formed ring-like and helical structures after incubation under low salt conditions. Electron micrographs of negatively stained MxA oligomers. Final protein concentrations were 0.05 mg/ml. A, recombinant MxA was diluted in HCB (see “Experimental Procedures”) with the indicated concentrations of NaCl and incubated on ice or at 37 °C for 10 min. B, recombinant MxA was diluted in HCB50 in the presence of the nucleotides indicated (200 μM each or 5 mM AlF₄⁻ in combination with 100 μM GDP) and incubated at 37 °C for 10 min before being prepared for electron microscopy. Scale bars, 150 nm (A and B). guanosine nucleotides leads to the formation of distinct MxA oligomeric structures.
with guanosine nucleotides under low salt condition. An increased oligomerization state could also be detected in the sedimentation assay when MxA was incubated with GDP/AlF$_4^-$, we could demonstrate that the effect of GDP/AlF$_4^-$ depends on a functional GTP-binding domain by using MxA(T103A), a mutant form of MxA that has no GTP binding capacity. AlF$_4^-$ binds to the GDP-loaded form of GTP-binding proteins thereby mimicking the conformation of a transition state, i.e. GDP-P$_\text{i}$ during hydrolysis of GTP (47). Therefore, the large aggregates of MxA stabilized in the presence of GDP/AlF$_4^-$ might reflect the conformational intermix during the transition from the GTP-loaded to the GDP-loaded molecule. Interestingly GDP/AlF$_4^-$ does not have the same effect on dynamin but forms extended spirals similar to those found with GTP/$\gamma$S (48). In the case of MxA, GTP/$\gamma$S and GDP/AlF$_4^-$ appear to stabilize different oligomeric states during MxA self-assembly. This is in agreement with previous findings indicating that GTP/$\gamma$S and GDP/AlF$_4^-$ block the GTPase cycle at different steps (47).

Small GTPases like p21ras have been shown to associate with AlF$_4^-$ in their GDP-bound form only in the presence of their respective GTPase-activating proteins (44). The strong effect of GDP/AlF$_4^-$ on MxA oligomerization supports the view that large GTPases such as Mx proteins and dynamins harbor an intrinsic GTPase-activating function and hence are able to hydrolyze GTP without additional cofactors (33–35). Homo-oligomerization into ring-like and helical structures appears to be essential for the biological function of dynamin-like proteins and their membrane fission activities (18, 36). What might be the function of MxA self-assembly? It was recently demonstrated that oligomerization stimulates the GTPase activity of MxA and prevents proteolytic degradation of the protein (49, 50). The MxA mutant MxA(L612K), having an amino acid exchange from leucine to lysine at position 612 within the leucine zipper motif, has lost both its GTPase activity and its ability to self-assemble into oligomers (30, 50, 51). This mutant is rapidly degraded when expressed in transfected cells, whereas wild-type MxA is stable (50). Therefore, we propose that the high molecular weight MxA oligomers found in interferon-treated human cells represent a storage form of MxA. Constant hydrolysis of GTP might induce the release of antivirally active MxA monomers from these intracellular storages.

We have further postulated that, in infected cells, the free MxA monomers bind to specific viral structures and subsequently oligomerize around their viral targets (50). Previous cosedimentation experiments with MxA and viral nucleocapsids demonstrated that only GTP-bound MxA was able to interact with viral targets, whereas nucleotide-free MxA or GDP-bound MxA was not (41). This led us to propose that GTP-MxA is in an interactive conformation that is able to recognize viral target structures and that this active conformation is stabilized by GTP/$\gamma$S (24). Once associated with the viral structure, oligomerization might stimulate the hydrolysis of GTP, which in turn would lead to a conformational change in the molecule that may have a deleterious effect on the viral target. The present data support such a scenario. MxA formed ring-like structures after incubation with GDP or GDP/$\gamma$S. These rings (of about 60 nm for the outer diameter and 40 nm for the inner ring diameter) resemble in dimension and appearance the ring-like structures formed by dynamin (38–40). Addition of GTP/$\gamma$S increased the oligomerization state of MxA, inducing the transition of the rings into spirals and stacks of interconnected rings. Therefore, we propose that MxA exists in three different conformational and functional states within the cell. MxA homo-oligomers may represent an inactive storage form, while MxA monomers are most likely antivirally active and able to form helical polymers around viral target structures. We are currently trying to elucidate whether virus infection quantitatively influences the ratio between these different states.

In conclusion, we have demonstrated that the human MxA GTPase is able to self-assemble into higher ordered oligomers under physiological conditions that may stabilize the protein within interferon-treated cells. We have further shown that nucleotide binding induces gross conformational changes, resembling those described for dynamin, supporting the view that MxA may function as a mechanochemical molecular machine after binding to viral target structures. Studies are now under way to confirm this concept for the action of MxA in virus-infected cells.

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