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The central interactive region of human MxA GTPase is involved in GTPase activation and interaction with viral target structures

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Abstract To define domains of the human MxA GTPase involved in GTP hydrolysis and antiviral activity, we used two monoclonal antibodies (mAb) directed against different regions of the molecule. mAb 2C12 recognizes an epitope in the central interactive region of MxA, whereas mAb M143 is directed against the N-terminal G domain. mAb 2C12 greatly stimulated MxA GTPase activity, suggesting that antibody-mediated crosslinking enhances GTP hydrolysis. In contrast, monovalent Fab fragments of 2C12 abolished GTPase activity, most likely by blocking intramolecular interactions required for GTPase activation. Interestingly, intact IgG molecules and Fab fragments of 2C12 both prevented association of MxA with viral nucleocapsids and neutralized MxA antiviral activity in vivo. mAb M143 had no effect on MxA function, indicating that this antibody binds outside functional regions. These data demonstrate that the central region recognized by 2C12 is critical for regulation of GTPase activity and viral target recognition.

Key words: MxA GTPase; Dynamin superfamily; Antiviral effect

1. Introduction

Mx proteins belong to the superfamily of dynamin-like GTPases. These differ from small GTPases and heterotrimeric G proteins in features such as their large size (70–100 kDa), a relatively low affinity for GTP, and a high intrinsic rate of GTP hydrolysis [1,2]. They contain a highly conserved tripartite GTP binding motif within the N-terminal G domain. The less conserved C-terminal part serves different functions like homooligomerization and association with partner molecules [3]. Homooligomerization seems to be essential for biological activity. Self-assembly of dynamin, for example, stimulates its GTPase activity [4] and is crucial for dynamin-mediated endocytotic processes [5–7]. Mx proteins also form homooligomers of high molecular weight [8,9]. Two distinct regions of human MxA, a central interactive region (amino acids 372–540) and a C-terminal leucine zipper motif (amino acids 564–662), are responsible for intra- and intermolecular interactions. This has been shown in previous experiments using a cotranslocation assay [10] or the yeast two-hybrid system [11]. In addition, biochemical studies with protease-digested MxA demonstrated that association of the C-terminal leucine zipper motif with the N-terminal half of the protein is required for GTP hydrolysis, indicating that the C-terminal region possesses GTPase-activating function [12]. A functional GTP binding motif was shown to be necessary for the antiviral activity of MxA [10,13]. We recently demonstrated a physical association of MxA with viral nucleocapsids of *Thogoto* virus (THOV) [14], an orthomyxovirus that is extremely sensitive to the antiviral effect of MxA [15]. Interaction of MxA with viral target structures was dependent on the presence of GTPγS, a non-hydrolyzable GTP analogue, indicating that the GTP-bound form of MxA has the antivirally active conformation [16]. Moreover, microinjection of nucleocapsids of THOV into MxA-expressing cells showed that MxA blocks the transport of these viral structures into the nucleus, the site of THOV replication [17].

Here, we investigated the involvement of defined regions of MxA in GTP hydrolysis and antiviral activity by utilizing two monoclonal antibodies (mAb). mAb M143 binds to a N-terminal epitope, whereas mAb 2C12 recognizes a central epitope of MxA. We show that preincubation of MxA with mAb 2C12 has a dramatic effect on the intrinsic GTPase activity, confirming that the central interactive region is involved in activation of GTP hydrolysis. The same antibody prevented cosedimentation of MxA with viral nucleocapsids and neutralized its antiviral activity in vivo. In contrast, mAb M143 had no effect on MxA function. These data indicate that the central 2C12 binding site of MxA is involved in regulation of both GTPase activity and viral target recognition within the infected cell.

2. Materials and methods

2.1. Antibodies

mAb M143 was produced in hybridoma cells from mice immunized with His-tagged MxA protein purified from *Escherichia coli* [13]. mAb 2C12 was raised against the murine Mx1 protein but cross-reacts with the human MxA protein [18,19]. mAb 1622 directed against the SV40 large T antigen was used as a control [20]. The monoclonal antibodies were purified from hybridoma cell culture supernatants by protein A-Sepharose chromatography. Fab fragments were prepared by digestion with immobilized papain (Pierce) and subsequent gel filtration using a Superdex-200-HR 16/60 column (Pharmacia). All antibody preparations were concentrated using Centricon 30 (Amicon) to a concentration of 2 mg/ml in 10 mM Tris–HCl, pH 7.5, 120 mM KCl.

2.2. Immunoprecipitation

MxA-expressing Swiss 3T3 cells (clone 4.5.15) [21] were metabolically labeled for 8 h by incubation with 50 μCi/ml [^{35}S]methionine in medium lacking methionine. Lysates were prepared in 50 mM Tris, pH 7.5, 0.1% Nonidet P-40, 5 mM $MgCl_2$, and 0.5 mM DTT. The lysates were mixed with 5 μg of purified intact IgGs and protein

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Abbreviations: GED, GTPase effector domain; mAb, monoclonal antibody; THOV, *Thogoto* virus

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A-Sepharose and incubated for 2 h at 4°C. Subsequently, the Sepharose beads were collected, and the bound ^{35}S-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

2.3. Isolation of His-tagged MxA and GTPase assay

Histidine-tagged MxA was produced in *E. coli* and isolated using Ni^{2+}-chelate chromatography as described [13]. MxA was further purified by Mono Q ion exchange chromatography in 20 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 10% glycerol, 300 mM NaCl [14].

GTPase activity was determined as described [9]. Briefly, 1 μg of purified MxA was incubated for 20 min at 37°C in 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.1 mM dithiothreitol, 100 nM AMP-PNP, 13 nM [α-^{32}P]GTP (3000 Ci/mmol), and 1 mM GTP. The reaction was terminated by the addition of SDS and EDTA to final concentrations of 0.5% and 2 mM, respectively. Nucleotides were separated by thin layer chromatography on polyethyleneimine–cellulose plates in 1 M LiCl and 1 M acetic acid. The signals were quantified by Phosphoimager (Fujix BAS1000, Fuji). To analyze the effect of the antibodies, 1 μg of purified MxA was preincubated with 1 μg of IgG molecules or Fab fragments for 20 min at 25°C prior to the GTPase assay.

2.4. Cosedimentation of MxA with viral RNPs in glycerol gradient centrifugation

Cosedimentation assays were performed as previously described [14]. Briefly, lysates of MxA-expressing cells (clone 4.5.15) were mixed with lysates of non-infected cells or THOV-infected cells in the presence of 200 μM GTPγS. The mixture was subjected to glycerol gradient centrifugation and the resulting fractions were analyzed by Western blotting using antibodies directed against MxA and THOV nucleoprotein. To assess the influence of mAbs, MxA cell lysates were treated with 6 μg of mAbs or Fab fragments before incubation with lysates of THOV-infected cells.

2.5. Microinjection of antibodies and virus infection

Swiss mouse 3T3 cells constitutively expressing MxA (clone 4.5.15) were seeded onto glass coverslips and antibodies (1 μg/μl) were microinjected into the cytoplasm of the cells as described [17]. Five hours later, the cells were infected with 50 plaque forming units of THOV per cell. After 16 h, the cells were fixed and analyzed by double immunofluorescence. Viral proteins were detected using a guinea pig anti-THOV antiserum and a corresponding TRITC-conjugated secondary antibody. DTAF-conjugated goat anti-mouse antibodies were used to detect the microinjected mouse IgGs.

3. Results

Two mAbs were used to characterize regions of MxA involved in GTP hydrolysis and antiviral activity. The first mAb, M143, was generated by immunizing mice with recombinant human MxA protein. The second mAb, 2C12, is directed against murine Mx1 protein and cross-reacts with human MxA [18,19]. Both antibodies recognized full-length MxA and immunoprecipitated the native protein from lysates of MxA-expressing cells (Fig. 1A). For further characterization, MxA fragments were fused to GST and expressed in *E. coli* [14]. mAb M143 recognizes an epitope in the N-terminal G domain of MxA (amino acids 1–363), whereas mAb 2C12 binds to a central region (amino acids 372–540) (data not shown, summarized in Fig. 1B). Using shorter fragments of rat Mx3, Johannes et al. determined the 2C12 binding site to be located between amino acids 430 and 469 of rat Mx3 [22]. This region is well conserved between Mx proteins and corresponds to amino acids 432–471 of human MxA (Fig. 1C, upper three lines).

3.1. Antibody 2C12 affects GTP hydrolysis of MxA

MxA has a tripartite GTP binding motif in its N-terminus (Fig. 1B). The importance of this motif for GTP binding and hydrolysis has been documented by mutational analysis [10,13]. Therefore, it was conceivable that binding of M143 to the N-terminal G domain of MxA would affect GTP hydrolysis. We performed GTPase assays in the presence of mAbs M143 or 2C12 (Fig. 2). Preincubation of recombinant MxA with IgGs or Fab fragments of M143 had no effect on GTPase activity (Fig. 2; M143) suggesting that this antibody binds outside the critical region involved in GTP binding and hydrolysis. In contrast, preincubation of MxA with monovalent Fab fragments of antibody 2C12 reduced GTPase activity approximately five-fold (Fig. 2; 2C12 (Fab)). Surprisingly, preincubation of MxA with whole IgG molecules of 2C12 had a stimulating effect on GTP hydrolysis (Fig. 2A; 2C12 (IgG)). Quantification of GDP production in these assays revealed a 2.6-fold increase in GTP hydrolysis as compared to the M143-treated or untreated controls (Fig. 2B). This unexpected result suggests that antibody-mediated crosslinking of MxA stimulates GTP hydrolysis.

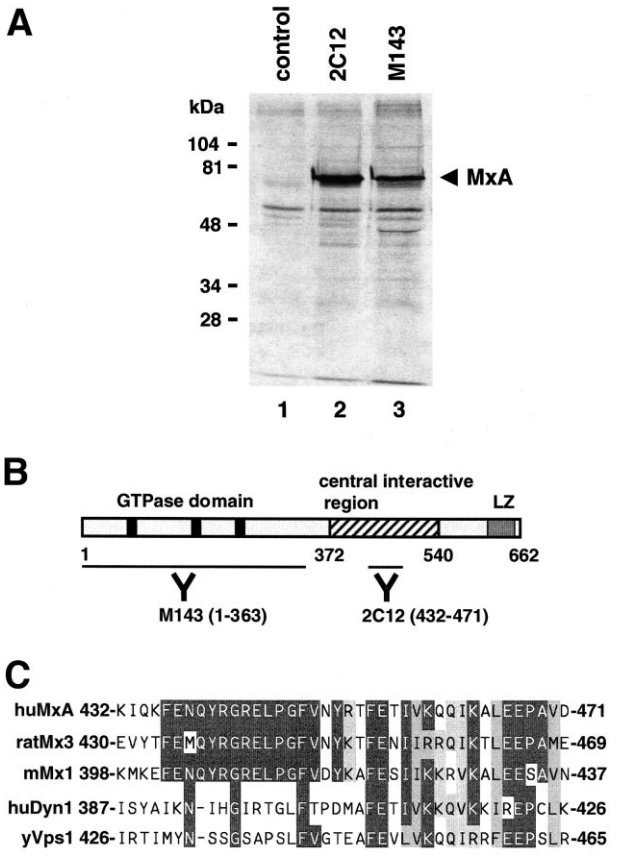

Fig. 1. Regions of MxA recognized by monoclonal antibodies 2C12 and M143. A: Immunoprecipitation of MxA by mAbs 2C12 and M143. ^{35}S-labeled extracts of MxA-expressing cells were incubated with antibodies 2C12 and M143, or with an irrelevant control antibody. The complex was precipitated using protein A-Sepharose beads. The labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. B: Domain structure of human MxA showing the N-terminal tripartite GTP binding region (black boxes), the central interactive region (striped box), and the C-terminal leucine zipper motif (LZ; gray box). The regions recognized by monoclonal antibodies M143 and 2C12 are indicated by black bars. C: Sequence alignment of the 2C12 binding region according to Johannes et al. [22]. The sequences of human MxA [29], rat Mx3 [30], mouse Mx1 [31], human dynamin-1 [32], and yeast Vps1 [33] are shown in the alignment. Identity is indicated by black boxes, and similarity by gray boxes.

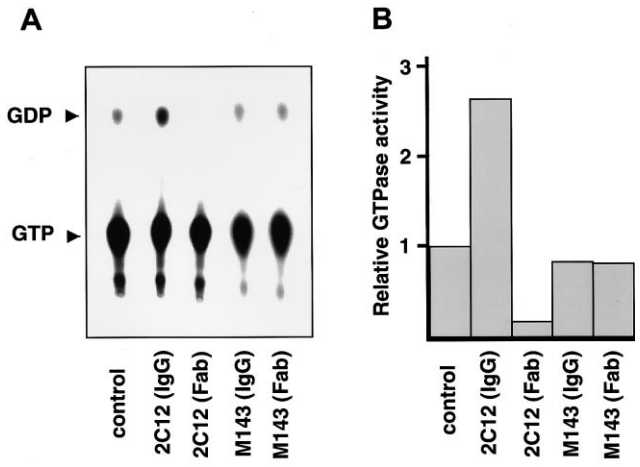

Fig. 2. Effect of monoclonal antibodies 2C12 and M143 on MxA GTPase activity. Recombinant His-MxA purified from *E. coli* (1 μg/assay) was incubated with radiolabeled GTP for 20 min at 37°C. MxA was preincubated with whole IgG molecules or Fab fragments (1 μg/assay) of mAbs M143 or 2C12, as indicated in Section 2. A: Autoradiograph of the reaction products after thin layer chromatography. B: Quantitative analysis of the GTPase activity was normalized to the activity of MxA alone.

3.2. Binding of MxA to viral nucleocapsids is prevented by mAb 2C12

We recently described an association of MxA with viral nucleocapsids [14]. When lysates of MxA-expressing cells were mixed with lysates of THOV-infected cells, a fraction of MxA cosedimented with the viral nucleocapsids into the glycerol gradient. In contrast, MxA remained at the top of the gradient in the absence of viral nucleocapsids (Fig. 3A,B). This interaction could be prevented by preincubation of MxA with mAb 2C12 (Fig. 3C) [14]. Because intact IgG and Fab fragments of mAb 2C12 had opposite effects on GTP hydrolysis, we compared the ability of these two antibody preparations to prevent binding of MxA to viral nucleocapsids. Interestingly, Fab fragments had the same effect as intact IgG molecules and abolished cosedimentation of MxA with the viral nucleocapsids (Fig. 3D). Pretreatment of MxA with IgG or Fab fragments of M143 did not interfere with nucleocapsid recognition. Thus, MxA continued to cosediment with the viral nucleocapsids to fractions of higher density (Fig. 3E,F). The different behavior of the two mAbs in the MxA cosedimentation assay suggests that the central interactive region of MxA is critically involved in nucleocapsid recognition and that binding of M143 to the N-terminal part does not affect this activity.

3.3. 2C12 but not M143 abolishes the antiviral activity of MxA

We next investigated the effect of intact antibodies and Fab fragments on the antiviral activity of MxA in living cells. Cells constitutively expressing MxA were microinjected with the different antibody preparations and subsequently infected with THOV. Viral proteins and microinjected antibodies were visualized by double immunofluorescence, using appropriate antisera. Intact IgGs of 2C12 neutralized the antiviral activity of human MxA (Fig. 4a,b), thus confirming previous results [17]. Cells microinjected with Fab fragments of 2C12 also became permissive for THOV infection (Fig. 4e,f), indicating that binding of the 2C12 fragments to MxA was sufficient to neutralize the antiviral effect of MxA. Likewise, 2C12 also neutralized the antiviral activity of MxA against influenza A virus and vesicular stomatitis virus (data not shown). These results demonstrated that the 2C12 binding site is crucial for the antiviral activity of MxA against all three viruses. In contrast, MxA-expressing cells microinjected with whole IgGs or Fab fragments of M143 did not produce viral proteins, indicating that the antiviral activity of MxA was unaffected (Fig. 4c,d,g,h).

4. Discussion

We used two mAbs to characterize domains of human MxA that are important for GTP hydrolysis, association with viral nucleocapsids and antiviral activity. mAb M143 had no effect in all three test systems. The antibody recognizes an epitope that is located within the N-terminal half of MxA (amino acids 1–363). This domain contains the sequence elements necessary for GTP binding [23,24]. Because M143 did not show any effect on MxA function, the antibody most likely binds to an epitope that is not involved in GTP binding and hydrolysis.

In contrast, mAb 2C12 had a strong effect on GTP hydrolysis, abolished the ability of MxA to associate with viral target structures, and neutralized its antiviral activity, indicating that the central interactive region recognized by 2C12 is crucial for MxA function. This region (amino acids 372–540; Fig. 1B) is involved in intra- and intermolecular interactions [10,14]. It has been proposed that the C-terminus containing the leucine zipper motif (amino acids 564–662) interacts with the central interactive region [11]. In fact, biochemical studies have shown that the last 98 C-terminal amino acids need to interact with the N-terminal core of the molecule to activate GTP hydrolysis [12]. Most likely, this intramolecular interaction of the C-terminus is blocked by binding of 2C12 Fab fragments to the central interactive region, leading to inhibition of GTP hydrolysis.

A GTPase-activating effect has also been described for the so-called GTPase effector domain (GED) of dynamin. This region (amino acids 618–752 of human dynamin-1) is able to stimulate GTPase activity of the N-terminal G domain of dynamin [6,25]. A yeast two-hybrid analysis suggests a direct interaction of GED with the so-called middle domain (amino acids 320–498) of dynamin [26] which reflects the results described for MxA [11]. Therefore, we compared the amino acid sequences of the middle domain of human dynamin-1 with the sequence of the 2C12 recognition site of various Mx proteins. Intriguingly, we found a cluster of mostly hydrophobic and basic amino acids that are highly conserved among these members of the dynamin-like GTPase superfamily, including a putative central interactive region of yeast Vps-1 (Fig. 1C). We assume that this region comprises the motif that interacts with the C-terminal leucine zipper motif of MxA (or the GED of dynamin) and is necessary for the GTPase-activating effect on the N-terminal G domain.

Why does the intact 2C12 antibody not have the same inhibitory effect on GTP hydrolysis as its Fab fragments? The simplest explanation is that intermolecular crosslinking of MxA to higher oligomeric structures by bivalent IgGs stimulates GTP hydrolysis. Aggregation of Mx oligomers could result in an interaction of C-terminal leucine zipper motifs with N-terminal G domains of neighboring molecules and

thereby enhances GTP hydrolysis. Interestingly, incubation of MxA with whole IgGs of M143 had no stimulating effect on GTP hydrolysis suggesting that the orientation of the crosslinked Mx molecules is critical. Crosslinking of MxA molecules via their N-termini by M143 most likely does not promote interactions between C-terminus and G domain.

In the case of dynamin, crosslinking by antibodies or by artificial templates such as microtubules also stimulates GTPase activity [4,27]. It has been proposed that activation of GTP hydrolysis in oligomeric structures is important for dynamin function, namely membrane fission [5–7,28]. These and the present data suggest that stimulation of GTP hydrolysis by the formation of high-ordered structures is in general a critical event in the function of dynamin-like GTPases.

Changes in the GTPase activity of Mx proteins may have a direct effect on their antiviral function. Mutational modifications within the G domain of MxA that abrogated GTP binding and hydrolysis abolished antiviral activity [10,13]. Therefore, we expected opposite effects of intact IgGs and Fab fragments of 2C12 on viral target recognition and inhibition of virus replication. Surprisingly, both 2C12 preparations blocked these activities, indicating that the influence of 2C12 on GTP hydrolysis and on antiviral activity is not directly correlated. Both 2C12 preparations inhibited the association of MxA with viral nucleocapsids suggesting either that the central interactive region is directly involved in this recognition event or, alternatively, that binding of 2C12 prevents the formation of an additional interactive domain necessary for nucleocapsid recognition. Coprecipitation experiments with MxA fragments showed that a complex structure is necessary for proper interaction of MxA with viral nucleocapsids [16]. The entire C-terminal half of MxA (amino acids 363–662) with the central interactive region and the C-terminal leucine zipper motif is required, whereas deletions of one or the other of these regions abolishes binding to nucleocapsids. Microinjection of 2C12 in MxA-expressing cells most likely blocks viral target recognition by a similar mechanism. A mutational analysis of the central interactive region of MxA is under way to identify the critical residues involved in these interactive events.

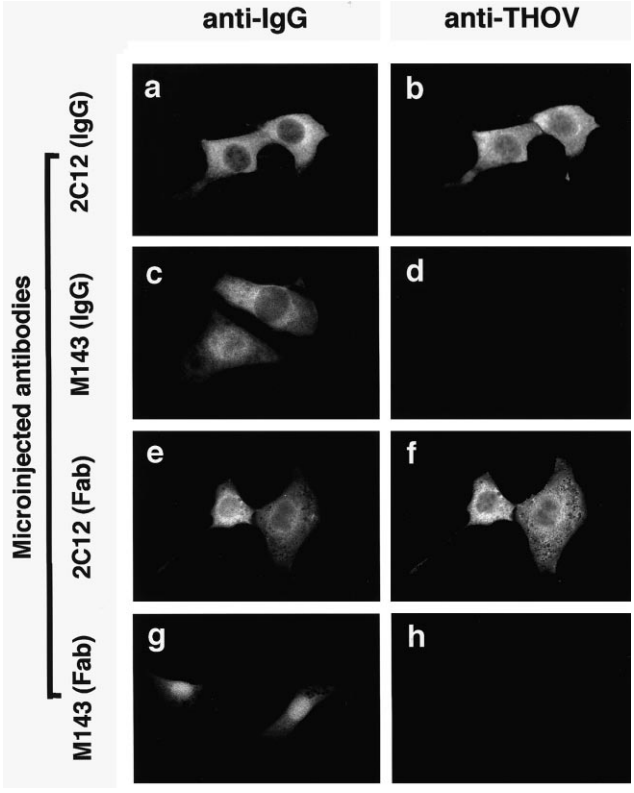

Fig. 4. mAb 2C12, but not M143, neutralizes the antiviral effect of MxA. MxA-expressing cells were microinjected with either intact IgGs of 2C12 (a,b) and M143 (c,d) or with Fab fragments of 2C12 (e,f) and M134 (g,h). The cells were infected with THOV, fixed 16 h later and analyzed by double immunofluorescence. Viral protein synthesis was detected using an anti-THOV antiserum (b,d,f,h) and the microinjected antibodies were visualized with a fluorescein-conjugated goat anti-mouse IgG antibody (a,c,e,g).

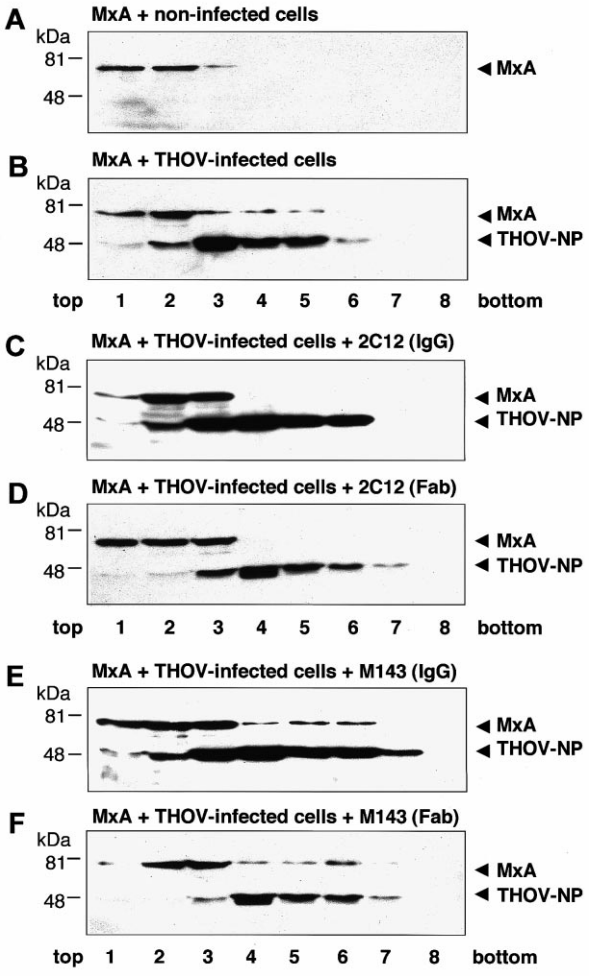

Fig. 3. Cosedimentation of MxA with nucleocapsids of THOV is prevented by mAb 2C12. Recombinant MxA was mixed with lysates of uninfected (A) or THOV-infected cells (B–F) in the presence of intact IgG molecules or Fab fragments as indicated (C–F). The mixtures were subjected to glycerol gradient centrifugation, and the resulting fractions were analyzed by Western blotting using antisera directed against MxA and viral NP (indicated by arrowheads).

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