Mx1 GTPase accumulates in distinct nuclear domains and inhibits influenza A virus in cells that lack promyelocytic leukaemia protein nuclear bodies

Othmar G. Engelhardt,1† Hüseyin Sirma,2 Pier-Paolo Pandolfi3 and Otto Haller1

1Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Hermann-Herder-Strasse 11, D-79104 Freiburg, Germany
2Heinrich-Pette-Institut für experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, D-20251 Hamburg, Germany
3Molecular Biology Program, Department of Pathology, Memorial Sloan-Kettering Cancer Center, Sloan-Kettering Institute, 1275 York Avenue, New York, NY 10021, USA

The interferon-induced murine Mx1 GTPase is a nuclear protein. It specifically inhibits influenza A viruses at the step of primary transcription, a process known to occur in the nucleus of infected cells. However, the exact mechanism of inhibition is still poorly understood. The Mx1 GTPase has previously been shown to accumulate in distinct nuclear dots that are spatially associated with promyelocytic leukaemia protein (PML) nuclear bodies (NBs), but the significance of this association is not known. Here it is reported that, in cells lacking PML and, as a consequence, PML NBs, Mx1 still formed nuclear dots. These dots were indistinguishable from the dots observed in wild-type cells, indicating that intact PML NBs are not required for Mx1 dot formation. Furthermore, Mx1 retained its antiviral activity against influenza A virus in these PML-deficient cells, which were fully permissive for influenza A virus. Nuclear Mx proteins from other species showed a similar subnuclear distribution. This was also the case for the human MxA GTPase when this otherwise cytoplasmic protein was translocated into the nucleus by virtue of a foreign nuclear localization signal. Human MxA and mouse Mx1 do not interact or form heterooligomers. Yet, they co-localized to a large degree when co-expressed in the nucleus. Taken together, these findings suggest that Mx1 dots represent distinct nuclear domains (‘Mx nuclear domains’) that are frequently associated with, but functionally independent of, PML NBs.

INTRODUCTION

Interferons (IFNs) induce the synthesis of a large number of proteins, some of which have antiviral activity (Der et al., 1998). Among these, the Mx proteins are best known for their capacity to inhibit the multiplication of many RNA viruses (for a recent review, see Haller & Kochs, 2002). Mx proteins are large GTPases with an intrinsic tendency to homooligomerize (Kochs et al., 2002; Melen et al., 1992; Nakayama et al., 1993). Different Mx proteins show different subcellular localizations and antiviral specificities. Interestingly, the subcellular compartment influences the antiviral spectrum to a great extent. The human cytoplasmic MxA protein inhibits members of the Orthomyxoviridae (such as the influenza viruses and Thogotovirus) and, in addition, a number of RNA viruses known to replicate in the cytoplasm. In contrast, the nuclear mouse Mx1 protein is active exclusively against orthomyxoviruses, known to have a nuclear replication phase (Haller et al., 1995; Staeheli et al., 1986). Moreover, when Mx proteins are translocated into a new compartment, they change their antiviral properties. For example, targeting of the cytoplasmic MxA protein to the nucleus by virtue of a foreign nuclear localization signal (NLS) changes the antiviral activity of MxA such that it still inhibits orthomyxoviruses but loses activity against all other MxA-sensitive viruses (Zurcher et al., 1992a). When the NLS of the nuclear Mx1 protein is inactivated by site-directed mutagenesis, the antiviral activity of the resulting cytoplasmic protein against influenza A viruses is lost. However, when this mutant Mx1 protein is equipped with a foreign NLS, it again
localizes to the nucleus and regains antiviral activity (Zurcher et al., 1992b). These results, among others, have led to the suggestion that the activity of Mx proteins depends on cofactors that are localized to specific subcellular compartments. We previously identified putative Mx-interacting proteins and demonstrated that these interaction partners localized to subnuclear structures known as promyelocytic leukaemia protein (PML) nuclear bodies (NBs) (Trost et al., 2000). We also found that mouse Mx1 forms characteristic dots in close spatial association with PML NBs (Engelhardt et al., 2001). These findings raised the intriguing possibility that PML NBs might contribute to the antiviral effect of Mx1.

PML NBs, also known as ND10, Kr bodies or PML oncogenic domains, are dynamic subnuclear structures. Their composition, number and size depend on various stimuli and vary during the cell cycle. In recent years, an increasing number of proteins have been found to reside within or to be partially associated with these structures, which are organized by PML as the main structural component (Negorev & Maul, 2001). PML NBs seem to have numerous functions (Borden, 2002; Piazza et al., 2001; Salomoni & Pandolfi, 2002; Zhong et al., 2000b). They appear to be involved in the regulation of transcription, in apoptosis and in cell-cycle regulation. Furthermore, they have been proposed to be storage depots where proteins not needed at a given time are kept inactive or modified until they are recruited to their sites of action (Negorev & Maul, 2001). Of particular interest is the role of PML NBs in viral infections. PML and other PML NB constituents such as Sp100 or ISG2 are inducible by type I IFNs (Gongora et al., 1997; Grotzinger et al., 1996; Guldner et al., 1992; Lavau et al., 1995; Nason-Burchenal et al., 1996; Regad & Chelbi-Alix, 2001) and it has been reported that PML itself has antiviral activity (Chee et al., 2003; Chelbi-Alix et al., 1998; Djavani et al., 2001; Regad et al., 2001). As a counter-measure, certain viruses seem to alter the composition and possibly the function of PML NBs. Thus, a number of DNA viruses associate with PML NBs and change their morphology (Everett, 2001). Less is known about possible interactions between RNA viruses and PML NBs. Infection with lymphocytic choriomeningitis virus leads to the relocation of the PML protein from the nucleus to the cytoplasm (Borden et al., 1998). Rabies virus expresses two proteins that interact with the PML protein and alter its distribution (Blondel et al., 2002). Interestingly, both these RNA viruses replicate in the cytoplasm but have the capacity to alter the composition of PML NBs in the nucleus. The exact role of these virus-host interactions is still unclear.

In the present study, we investigated the role of PML NBs in the nuclear localization of Mx1 and its antiviral activity against influenza A virus. We found that cells lacking PML NBs supported influenza virus growth to the same degree as wild-type cells, indicating that PML NBs are not involved in virus multiplication. Moreover, the intranuclear localization of Mx1 was unchanged in these cells. Likewise, nuclear Mx proteins of different species co-localized with murine Mx1 when co-expressed, indicating that Mx proteins prefer particular subnuclear domains, which may constitute specific ‘Mx nuclear domains’. Finally, the antiviral activity of Mx1 against influenza A virus was unchanged in cells lacking PML NBs.

METHODS

Cells and viruses. Mouse embryo fibroblasts (MEFs) were derived from PML−/− animals or control wild-type animals at day 13-5 of gestation and were kept in Dulbecco’s modified Eagle’s medium (DMEM) plus 20% FCS. Both types of cell are Mx1 negative for genetic reasons (Staeheli et al., 1988). Primary MEFs or MEFs immortalized by spontaneous transformation were used, as indicated. Mouse 3T3 cells, African green monkey VeroCH cells, human H1299 lung carcinoma cells, human HeLa and human 293T cells were maintained in DMEM plus 10% FCS. Influenza A virus M-TUR is derived from the avian A/Turkey/England 63 (H7N3) Langham strain and has been adapted to grow in mouse peritoneal macrophages (Lindemann et al., 1978). Virus stock was grown on PML−/− MEFs. Influenza A virus FPV-B, a mammalian cell-adapted variant of influenza A/FPVDobson/34 (H7N7), was grown on Swiss 3T3 cells (Israel, 1979).

Antibodies. Monoclonal anti-MxA antibody M143 recognizes a conserved N-terminal epitope and shows broad cross-reactivity to Mx proteins from many species, including mouse Mx1 (Floh et al., 1999). Polyclonal rabbit anti-Mx1 antibody is a hyperimmune serum of rabbits immunized with highly purified recombinant Mx1 protein produced in E. coli. Polyclonal anti-Influenza A virus hyperimmune rabbit serum has been described previously (Haller et al., 1976). Polyclonal rabbit antiserum against Daxx (M-112), polyclonal rabbit (H-238) and goat (A-20) antisera against PML and polyclonal rabbit antisera against the myc epitope (A-14) were obtained from Santa Cruz Biotechnologies. Anti-FLAG tag (M2) and anti-SC35 monoclonal antibodies were from Sigma-Aldrich. Monoclonal mouse (5P10-p) and polyclonal rabbit anti-p80 collagen antibodies were kind gifts from Werner Franke (DKFZ, Heidelberg, Germany) and Angus Lamond (Wellcome Trust Biocentre, University of Dundee, Dundee, UK), respectively. Secondary antibodies used were donkey anti-mouse IgG, donkey anti-rabbit IgG and donkey anti-goat IgG conjugated to Cy2, Cy3 or Cy5, as required (Jackson ImmunoResearch Laboratories).

Plasmids. Plasmids pHMG-Mx1 (Kolb et al., 1992), pHMG-Mx1(K49A) (Pitossi et al., 1993), pHMG-Mx1(K49M) (Pitossi et al., 1993) and pHMG-FLAG-MxA (Ponten et al., 1997) have been described previously. Plasmid pHMG-FlagMx1 was kindly provided by Georg Kochs (Department of Virology, University of Freiburg, Freiburg, Germany). Briefly, the FLAG sequence was cloned into the pSp65-Mx1(3’-CGGTACCGAAGATGGACTACAAGGACGACGATGA-3’ I restriction site of pSP65-Mx1(ClaI) (Zurcher et al., 1992b); the resulting FLAG–Mx1 cDNA was transferred into the pMal restriction site of pSPl65-Mxl(ClaI) (Zurcher et al., 1992b); the resulting FLAG–Mx1 cDNA was transferred to plasmid pHMG (Gautier et al., 1989). The resulting construct pHMG-FlagMx1 carried the FLAG sequence as a 5’-terminal extension of the Mx1 open reading frame. Plasmid pHMG-rat Mx1 was produced by inserting the rat Mx1 cDNA (Meier et al., 1992b); the resulting FLAG–Mx1 cDNA was transferred to plasmid pHMG (Gautier et al., 1989). pEGFP-Mx1 was kindly provided by Jovan Pavlovic (Institute for Medical Virology, University of Zürich, Zürich, Switzerland), pEGFP-PML was a kind gift from Hans Will (Heinrich-Pette-Institut, Hamburg, Germany). Plasmid pCImycTMxA was generated by replacing the BamH1–Nol fragment of plasmid pCImycPKM-C1 (Engelhardt et al., 2003) with a PCR product containing the open reading frame of TMxA (Zurcher et al., 1997).
Transfections and transductions. Cells were seeded into six-well plates or 24-well plates (with or without glass coverslips) and transfected the following day using Lipofectamine (Life Technologies) according to the manufacturer’s instructions.

For transfection with retroviral vectors, cells were seeded into 24-well plates, washed with PBS the next day and incubated with retroviral vector-containing supernatants for 4–6 h in the presence of 8 µg polybrene ml⁻¹ (Sigma-Aldrich). More virus-containing supernatant was added and cells wereincubated at 37°C overnight. The inoculum was removed and replaced with DMEM plus 20% FCS. Cells were processed for immunofluorescence staining or infection by influenza A virus (see below) 3–6 days post-transfection.

Antiviral test. PML⁻/⁻ or control wild-type MEFs were seeded into the wells of a 24-well plate and transfected the next day with retroviral vectors expressing Mx1, Mx1(K49A) or Mx1(K49M). The cells were split 3 days later into a 24-well plate containing glass coverslips, infected the following day with influenza A virus M-TUR and fixed 6 h post-infection in 3% paraformaldehyde. Immunofluorescence staining was carried out as described below with a polyclonal rabbit anti-influenza A virus serum and monoclonal anti-Mx antibody M143. For quantification, cells expressing Mx1 or mutant Mx1 proteins and influenza A virus antigens and cells expressing only Mx1 proteins without influenza A virus antigens were counted to determine the infection rate of Mx-expressing cells. The infection rate of cells not expressing Mx1 proteins on the same coverslips was determined in parallel.

Immunofluorescence staining and confocal microscopy. Immunofluorescence staining was carried out as described previously (Engelhardt et al., 2001). In some experiments, DNA was counterstained by incubation in Bisbenzimide (0.33 mg ml⁻¹ in methanol; Sigma-Aldrich) for 10 min or by incubation in TO-PRO-3 iodide (diluted 1:1000; Molecular Probes) for 30 min. Coverslips were mounted in Mowiol (Sigma-Aldrich) containing DABCO [1,4-diazabicyclo(2.2.2)octane; Sigma-Aldrich].

Analysis was done on a Leica DM-IRBE microscope using a 63× objective and appropriate filter sets (Leica Microsystems). Confocal microscopy was performed on a Leica TCS SP2 attached to a Leica DM-IRBE microscope with a 63× objective and on a Radiance 2000 confocal microscope (Bio-Rad) attached to a Nikon Eclipse TE300 microscope with a 60× objective. Image acquisition was done using the appropriate proprietary confocal software, recording the different channels separately. Figures were assembled using Adobe Photoshop, Adobe Illustrator and MetaMorph (Universal Imaging Corporation).

Distance measurements and statistical analysis. Confocal images of single planes were processed separately for each channel using MetaMorph software. Images were smoothed by applying a median filter, followed, where necessary, by conversion to binary images, pixel erosion and segmentation. Finally, the coordinates of the centroids of the individual domains were exported to Microsoft Excel. For each centroid of PML, SC35 or coilin, the distance to the nearest Mx1 dot was calculated. At least 30 nuclei for each set of labellings were used for statistical analysis. Mean minimum distances, calculated as the mean of all minimum distances determined in one experiment, were compared using Student’s t-test. In the case of coilin–Mx1 measurements, the data were further split. The mean of all per-cell means of the minimum PML–Mx1 distances was used as an arbitrary threshold value to classify the degree of association between PML and Mx1; cells with a per-cell mean minimum distance below this threshold were termed ‘high PML–Mx1 association’, and all others ‘low PML–Mx1 association’. Means of minimum coilin–Mx1 distances were determined for each of these two groups and compared using Student’s t-test. Differences were considered statistically significant for P < 0.01.

RESULTS

Influenza A virus growth in PML-deficient cells

PML has been reported to exhibit antiviral activity against influenza A virus (Chelbi-Alix et al., 1998), while a number of DNA viruses appear to replicate and/or transcribe in the vicinity of PML NBs (Everett, 2001). We therefore tested whether influenza A virus would grow normally in cells lacking PML. PML-deficient and wild-type MEFs were infected with 0·0005 p.f.u. influenza A virus strain FPV per cell and supernatants were titrated at various times after infection to determine the amount of progeny virus produced. Fig. 1 shows the growth curves obtained with the two different cell types. At each time point analysed, the virus yields in PML-deficient and wild-type cells were comparable, indicating that the presence or absence of PML was irrelevant for virus growth in these MEFs.

![Fig. 1. Growth of influenza A virus in cells lacking PML NBs. Spontaneously transformed MEFs from wild-type (▲) and PML⁻/⁻ (■) mice were infected with 0·0005 p.f.u. influenza A virus strain FPV-B per cell. At various times after infection (h.p.i.), supernatants were harvested and titrated by plaque assay on VeroCH cells. Growth curves were determined in triplicate and mean titres ± SD are shown.](http://vir.sgmjournals.org)
Mx1 localization in cells lacking PML NBs

PML−/− mice are derived from the inbred strain 129Sv known to carry a natural defect at the Mx1 gene locus on chromosome 16 (Staeheli et al., 1988). Hence, these animals have a defective Mx1 gene in addition to the targeted disruption of the PML gene. We therefore used transient
Introduction of PML protein into PML
Mx1 was not directly associating with Daxx (Fig. 2g–i). localizing close to accumulations of Daxx, suggesting that whether the expression of PML protein in PML or Sp100, relocalize to these domains. We therefore tested Zhong et al leads to the reformation of PML NBs. (a)–(c) MEFs from wild-type (a–c) or PML genetic Mx1-positive mouse strains, Mx1 was frequently found juxtaposed to and partially over-
lapping PML NB marker proteins. In PML was frequently found juxtaposed to and partially over-
lapping PML NBs (Fig. 2a–c, right cell), as previously described (Chelbi-Alix et al., 1995; Engelhardt et al., 2001). Low levels of Mx1 led to a more fine-punctate nuclear distribution and little association with PML NBs (Fig. 2a–c, left cell). When PML Mx1 protein was found either diffusely in the nucleus (Fig. 2e) or in patchy, diffuse accumulations (Fig. 2h), many of which coincided with patches of DNA staining (Ishov et al., 1999, and data not shown). Examination of double-labelled PML Mx1 was also found associated with PML NBs. Indeed, Mx1 was also found associated with PML NBs. Nevertheless, these Mx1 dots are localized close to PML NBs, once these structures are reformed in PML−/− cells.

Mx1 protein has antiviral activity in the absence of PML NBs
We assessed whether PML NBs were required for the antiviral activity of Mx1. Wild-type and PML−/− cells were transduced with lentiviral vectors expressing Mx1 and were subsequently infected with influenza A virus. The cells were fixed 5–6 h after infection and processed for immunofluorescence analysis, using specific antibodies against Mx1 and viral proteins. The infection rate was quantified as indicated in Methods. As controls, two antivirally inactive mutant forms of Mx1, namely Mx1(K49M) and Mx1(K49A) (Pitossi et al., 1993), were expressed and analysed in parallel. Fig. 3(a) shows that Mx1 protected both wild-type and PML−/− cells from infection. As expected, Mx1(K49A) had no antiviral effect in either cell culture. Fig. 3(b) shows the quantification of a representational experiment. Mx1 inhibited infection of PML-negative cells to the same degree as wild-type cells, while the mutant proteins Mx1(K49M) and Mx1(K49A) had no effect. Clearly, Mx1 inhibited influenza A virus in the absence of PML, indicating that its antiviral function was not dependent on PML NBs.

Nuclear Mx proteins localize to distinct subnuclear domains
Rat Mx1 protein is found in a dot-like pattern similar to mouse Mx1 in the nucleus of IFN-treated cells (Meier et al., 1988). We transfected an expression plasmid for rat Mx1 into wild-type MEFs and performed double-immunofluorescence analysis to check whether or not rat Mx1 was also found associated with PML NBs. Indeed, staining with anti-Daxx antibodies revealed that rat Mx1 preferentially accumulated in the immediate vicinity of PML NBs, exactly like mouse Mx1 (Fig. 4a–c). More importantly, rat Mx1 retained its ability to form nuclear dots in PML−/− cells (Fig. 4d–f).

![Fig. 2.](image-url) Mx1 nuclear dots in the absence of PML NBs. (a)–(i) MEFs from wild-type (a–c) or PML−/− (d–i) mice were transduced with a lentiviral vector expressing Mx1 protein, fixed 6 days later and immunostained with a polyclonal rabbit antiserum against Daxx protein (green) and a mouse monoclonal antibody detecting Mx1 protein (red). The red and green channels were recorded separately by confocal microscopy and superimposed electronically (merge). (a)–(c) Two Mx1-expressing wild-type cells. The right cell shows the typical Mx1 dots. The left cell expressed small amounts of Mx1 and shows little association of Mx1 with Daxx. (d)–(f) An Mx1-expressing cell with fine-punctate and diffuse Daxx on a PML−/− background. (g)–(i) Cell with patchy accumulations of Daxx. (j)–(q) Wild-type MEFs (j–m) and PML−/− MEFs (n–q) were transfected with expression plasmids pHMG-Mx1 and pEGFP-PML, fixed 2 days later and stained with primary antibodies as above. GFP–PML was visualized by its autofluorescence. Red, green and blue channel images were recorded separately and overlaid electronically (m, q). Inserts in (m) and (q) show the overlay of the red and green images of a part of the image [the lower Mx1-positive cell in (m) and the upper cell in (q)]. Bars, 8 μm.
These findings suggested that specific subnuclear domains exist in which Mx proteins accumulate when targeted to the nucleus. To verify this, we used a nuclear form of MxA as a marker. Normally, the human MxA protein accumulates in the cytoplasm of cells (Staeheli & Haller, 1985), where it is partly associated with membranes of the smooth endoplasmic reticulum (Accola et al., 2002). It is known to form homooligomers but not heterooligomers with other Mx proteins, such as human MxB (Melen & Julkunen, 1997). MxA has been shown to translocate from the cytoplasm to the nucleus when equipped with the NLS of the SV40 large T antigen (Zurcher et al., 1992a). This nuclear MxA protein, called TMxA, showed a dot-like accumulation pattern in the nucleus (Ponten et al., 1997; Zurcher et al., 1992a). To analyse its subnuclear localization in more detail, we co-expressed human TMxA with mouse Mx1 and analysed their respective localization. To avoid complications arising from antibody cross-reactivity, we used tagged versions of Mx proteins. Mouse Mx1 was tagged at its N terminus with a FLAG tag, whereas TMxA was expressed with an N-terminal myc tag. Both tagged proteins showed nuclear localization patterns indistinguishable from their untagged wild-type counterparts (data not shown). When cells were co-transfected with the two expression plasmids and double labelled with anti-FLAG and anti-myc tag antibodies, a high degree of co-localization of the two Mx proteins was observed (Fig. 5a–f). Frequently, dots containing both FLAG–Mx1 and myc–TMxA were found close to PML.
NBs (data not shown), suggesting that they were equivalent to Mx1 or TMxA domains in cells expressing these proteins individually.

To demonstrate that this co-localization was not simply due to heterooligomerization between the human and the mouse Mx proteins, we used a co-translocation assay that reveals oligomerization of Mx proteins in living cells (Kochs et al., 1998; Ponten et al., 1997). When nuclear TMxA and cytoplasmic MxA are co-expressed, they form homooligomers and translocate into the nucleus together (Ponten et al., 1997). We therefore asked whether the nuclear mouse Mx1 protein would be able to drag the human MxA into the nucleus. We transfected an expression plasmid encoding the GFP–Mx1 fusion protein, together with an expression plasmid encoding FLAG–MxA. In cells co-expressing these two Mx proteins, GFP–Mx1 accumulated in the characteristic nuclear Mx1 dots, whereas FLAG–MxA remained in the cytoplasm (Fig. 5g–i). These findings suggested that the co-localization of FLAG–Mx1 and myc–TMxA described above (Fig. 5a–f) was most likely not the result of oligomerization of the two proteins, although we cannot rule out the remote possibility that the nuclear environment favours Mx1–TMxA interactions that do not occur elsewhere in the cell. To prove that GFP–Mx1 was indeed acting like wild-type Mx1 in this assay, we co-expressed GFP–Mx1 and FLAG–MxA in cells. We found perfect co-localization of the GFP and FLAG signals (Fig. 5j–l). Moreover, GFP–Mx1 accumulated in nuclear dots that were frequently associated with PML NBs and inhibited influenza A virus (data not shown).

We also analysed the relationship of Mx1 dots with other prominent nuclear structures, namely SC35 speckles and Cajal bodies. Vero cells transfected with the plasmid expressing GFP–Mx1 were labelled with anti-PML and anti-SC35 or with anti-PML and anti-p80 coilin antibodies and examined by confocal microscopy. As expected, PML and GFP–Mx1 were frequently found closely associated (Fig. 6a–d). It should be noted that Vero cells without IFN treatment have a fairly small number of PML NBs per nucleus. SC35 speckles were often separated from Mx1 dots; however, a number of SC35 speckles displayed association with Mx1 dots and/or PML NBs (Fig. 6a and b and enlargements a1–b2). Similarly, Cajal bodies, as revealed by labelling p80 coilin, were sometimes in close proximity to PML NBs as well as to Mx1 dots, whereas in other instances they occupied distinct areas of the nucleus (Fig. 6c, d). This varied pattern of localization and association prompted us to analyse the distances between these nuclear structures statistically, similar to previously reported approaches (Shiels et al., 2001; Wang et al., 2004). The distance between each PML, SC35 or coilin domain and their nearest Mx1 dot was determined as described in Methods. The mean minimum SC35–Mx1 distance was larger than the mean minimum PML–Mx1 distance (Fig. 6e), confirming our qualitative observation that Mx1 dots were associated more closely with PML NBs than with SC35 speckles. In contrast, the mean minimum distance between coilin and Mx1 dots did not differ significantly from the mean PML–Mx1 distance. Since the association of Mx1 with PML NBs depends, at least in part, on expression levels of Mx1 and its accumulation in bigger dots (see, for

![Fig. 4. Different nuclear Mx proteins display similar localization patterns. Wild-type (a–c) and PML−/− (d–f) MEFs were transfected with plasmid pHMG-ratMx1 expressing the rat Mx1 protein, fixed 1 day later and stained for immunofluorescence with a polyclonal rabbit antiserum against Daxx (green) and a monoclonal anti-Mx antibody (red). Red and green channel images were recorded separately and overlaid electronically. Bars, 8 μm.](http://vir.sgmjournals.org)
example, Fig. 2a–c), we divided the distance data for Cajal bodies into two groups, nuclei with 'low' and 'high' association between PML and Mx1 (see Methods for details). Comparison of the mean minimum coilin–Mx1 distances between these two groups revealed that the association of Cajal bodies with Mx1 dots reflected the degree of association between PML NBs and Mx1 dots, in as far as the mean minimum coilin–Mx1 distance was significantly lower in cells considered 'high PML–Mx1 association'.

DISCUSSION

In this study, we addressed the question of whether PML NBs determine the subcellular localization, antiviral activity, or both, of the IFN-induced Mx1 GTPase. We previously identified several proteins including Daxx, Sp100, SUMO-1, BLM, TOPORS and PKM/HIPK-2 as putative cellular interaction partners of Mx1 (Engelhardt et al., 2001; Trost et al., 2000). These proteins are known components of PML NBs (Negorev & Maul, 2001; Rasheed et al., 2002). Furthermore, PML, as well as Sp100, is also upregulated by IFN (Chelbi-Alix et al., 1995; Grotzinger et al., 1996; Guldner et al., 1992; Lavau et al., 1995; Nason-Buchanen et al., 1996) and PML itself has been reported to possess antiviral activity, like Mx1 (Chelbi-Alix et al., 1998; Djavani et al., 2001; Regad et al., 2001). Therefore, the intriguing possibility existed that Mx1 might take part in the complex interplay of PML NB constituents. We have shown here...
that in PML−/− cells Mx1 displayed a pattern of nuclear dots that was indistinguishable from the pattern observed in wild-type cells. Moreover, Mx1 had antiviral activity in PML−/− cells, indicating that PML and intact PML NBs are dispensable for the antiviral action of Mx1. Nevertheless, our findings also show that Mx1 preferentially localized to the vicinity of PML NBs, provided they were reconstituted in PML−/− cells.

It should be noted that the association of PML NBs and Mx1 is rarely complete. In most cells, only a subset of Mx1 dots is juxtaposed to or partially overlapping with PML NBs. Furthermore, cells expressing low levels of Mx1 often show a fine-punctate distribution of Mx1 with little or no obvious association with PML NBs, suggesting that expression levels determine localization. Thus, in IFN-treated cells, Mx1 may first accumulate in the form of small aggregates that fuse and grow bigger when protein levels increase. Larger aggregates may then form at particular sites in the nucleus that are found frequently in the immediate vicinity of PML NBs.

In cells lacking PML NBs, the sites of Mx1 dot formation seem to be preserved. It remains to be seen whether Mx1 interacts with other proteins in these areas. Protein–protein interactions in PML NBs and probably other subnuclear domains are highly dynamic. Daxx, Sp100, SUMO-1, TOPORS, FLASH, PIAS-1 and PKM/HIPK-2 are partially associated with a subset of Mx1 domains and may be engaged in such dynamic Mx1 interactions (Engelhardt et al., 2001; Trost et al., 2000; this study and data not shown).
An intriguing observation was the finding that nuclear Mx proteins of three different species exhibited an almost identical subnuclear distribution. The nuclear Mx1 protein of the rat behaved like mouse Mx1 and displayed dots that were frequently associated with PML NBs. Surprisingly, the human MxA protein, known to reside in the cytoplasm, adopted an Mx1-like localization upon artificial translocation into the cell nucleus. This nuclear form of MxA co-localized with mouse Mx1 in PML-associated dots and tracks. This co-localization was unexpected because the two proteins do not interact, as shown in an in vivo assay. Obviously, nuclear Mx proteins accumulate in their own distinct nuclear domains, which we have tentatively called ‘Mx nuclear domains’. These domains are functionally independent of PML NBs, albeit often closely associated with PML NBs in wild-type cells. We propose that the same protein–protein interactions leading to dot formation of mouse Mx1 in the vicinity of PML NBs also apply to other nuclear Mx proteins, including the nuclear form of MxA.

We observed that ‘Mx nuclear domains’ were not exclusively associated with PML NBs, but could also be found in the vicinity of Cajal bodies and, to a lesser degree, of SC35 speckles. Association of one nuclear domain with more than one type of other nuclear structure has been reported in the literature (Wang et al., 2002) and may be a consequence of the highly dynamic nature of most nuclear structures or of the multitude of protein–protein interactions of which some proteins are capable. The putative interaction partners of Mx1 are mostly components of PML NBs, suggesting a biochemical link of Mx1 to PML NBs. However, it remains possible that proteins will be found that link Mx1 to other nuclear structures. It is noteworthy that, in the natural situation of virus infection, Mx1 is induced by IFN, which also leads to an increase in the number of PML NBs but not of SC35 speckles and Cajal bodies, resulting in the high degree of association seen in mouse cells upon IFN treatment (Engelhardt et al., 2001).

An interesting question is whether dot formation is essential for Mx1 activity. It is possible that nuclear Mx proteins require the dot-forming domains in order to exert their antiviral activity against influenza viruses and other orthomyxoviruses. The sites of virus transcription and replication in the nucleus are presently not known. It is conceivable that these viral processes occur at or near the ‘Mx nuclear domains’ described here. Alternatively, Mx1 dots may serve as storage depots for excess Mx1 protein. This would imply that active Mx1 is recruited from these depots to sites of virus transcription and replication. A similar depot model has also been proposed for PML NBs (Negorev & Maul, 2001). If the sole purpose of Mx nuclear domains is to serve as storage depots, these domains are expected to be dispensable for antiviral activity. This is in line with previous findings showing that Mx1(C71S), a mutant form of Mx1 with a single amino acid exchange at position 71, is antiviral, although this mutant does not form the characteristic nuclear dots (Toyoda et al., 1995). However, using our high-expression vectors, this Mx1 mutant was able to accumulate in nuclear dots in a majority of cells (see Supplementary Figure in JGV Online), suggesting that dot formation most likely depends on protein expression levels, as discussed above. All other mutants of Mx1 with a diffuse intranuclear distribution are reported not to have antiviral activity (Garber et al., 1993). On the other hand, some inactive variants of Mx1 accumulated in dots in the nucleus (Pitossi et al., 1993) (see also Fig. 3a). Therefore, it is presently not possible to decide whether or not Mx1 dots play a direct role in the antiviral activity of Mx1. Further mutational studies will be needed to clarify this issue.

Our present study does not support the view that PML itself has antiviral activity against influenza viruses (Chelbi-Alix et al., 1998). In our hands, both wild-type and PML−/− cells were equally permissive for the influenza A virus strain used. This also suggests that PML NBs do not play an essential role in the influenza virus life cycle. In contrast, Mx1 was clearly antiviral, even in the absence of PML. Mx GTPases are members of the dynamin superfamily of large GTPases and may have specialized cellular functions in addition to their antiviral activity (Haller & Kochs, 2002). It will be interesting to define further the significance of the ‘Mx nuclear domains’ described here for the cellular and antiviral functions of this class of nuclear GTPases.

ACKNOWLEDGEMENTS

We thank Hans Will, Thomas Hofmann, Jovan Pavlovic, Angus Lamond and Werner W. Franke for reagents, Georg Kochs for plasmids and helpful discussions and Karin Engelhardt, Peter Staeheli, Friedemann Weber and Anneke Funk for critically reading the manuscript. This work was in part supported by a grant from the Deutsche Forschungsgemeinschaft to O. H. (HA 1582/3-1) and the BMBF (H. S.). The Heinrich-Pette-Institut is supported by the Freie und Hansestadt Hamburg and the Bundesministerium für Gesundheit und Soziale Sicherheit.

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