West Nile virus-induced cytoplasmic membrane structures provide partial protection against the interferon-induced antiviral MxA protein

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The human MxA protein is a type I and III interferon (IFN)-induced protein with proven antiviral activity against RNA viruses. In this study, we investigated the effect of MxA expression on the replication of West Nile Virus strain Kunjin (WNVKUN). Pretreatment of A549 cells with IFN-α lead to increased expression of MxA, which contributed to inhibition of WNVKUN replication and secretion. However, in Vero cells stably expressing the MxA protein, WNVKUN replication, maturation and secretion was not inhibited. Biochemical and subcellular localization studies of WNVKUN proteins and MxA suggest that the MxA activity was not compromised by a flavivirus-encoded antagonist. Instead, we show that characteristic membranous structures induced during WNVKUN replication provide partial protection from MxA, possibly by ‘hiding’ WNVKUN replication components. This distinct compartmentalization of viral replication and components of the cellular antiviral response may be an evolutionary mechanism by which flaviviruses can hide from host surveillance.

Kunjin strain of West Nile virus (WNVKUN) is a member of the Flaviviridae, a family of mosquito-borne, enveloped, positive-strand RNA viruses that cause many thousands of deaths each year. WNVKUN is endemic within Australia and is a causative agent of Australian encephalitis, but is seldom associated with severe disease (Mackenzie et al., 1994). Sequencing analyses have revealed that WNVKUN is closely related to WNV strain New York 99 (WNVNY99), the causative agent of the 1999 epidemic of encephalitis in New York City. Comparison between the nucleotide and amino acid sequences of WNVKUN and WNVNY99 revealed a very close phylogenetic relationship (88 and 98.1%, respectively) (Lanciotti et al., 1999; Liu et al., 2003; Shi et al., 2002).

The interferon (IFN)-dependent immune response appears essential for the protection and clearance of most viral infections, including flaviviral infections (Diamond & Harris, 2001; Lobigs et al., 2003; Samuel & Diamond, 2005). IFNs are inducible cytokines and their antiviral effect is mediated by several IFN-induced proteins; some prime examples of these are double-stranded RNA (dsRNA)-dependent protein kinase R (PKR), 2’-5’ oligoadenylate synthetases (OAS) and MxA (Goodbourn et al., 2000).

The Mx proteins belong to the dynamin superfamily of large GTPases, which are involved in different functions within the cell, including host defence (Haller & Kochs, 2002). MxA is induced by type I (IFN-α and IFN-β) and III (IFN-γ) IFNs via an autocrine feedback mechanism that initiates upregulation of MxA at the level of RNA transcription. MxA functions as a potent antiviral protein both in vitro and in vivo (Haller & Kochs, 2002; Holzinger et al., 2007). The antiviral activity of MxA appears to be based on recognition of pre-formed or forming viral nucleocapsids (NC) or at early steps of viral RNA transcription, perhaps uncoating (Marschall et al., 2000; Pavlovic et al., 1990, 1992; Reichelt et al., 2004; Schwemmle et al., 1995; Zhao et al., 1996). It has been well documented that MxA can inhibit the replication of many negative-strand RNA viruses and additionally at least two positive-strand RNA viruses (Chieux et al., 2001; Haller & Kochs, 2002; Landis et al., 1998), and recently two dsRNA viruses (Mundt, 2007), suggesting that the production of MxA is a broad-spectrum approach in providing an antiviral state.

As the IFN system is essential for flavivirus clearance (Diamond & Harris, 2001; Lobigs et al., 2003; Samuel & Diamond, 2005), we first analysed the inhibitory effect of the type I and II IFNs on WNVKUN infection in A549 cells (Fig. 1a, b). Cells were pretreated for 6 h with 1000 IU ml⁻¹ IFN-α (Roche) or 100 ng ml⁻¹ IFN-γ (Sigma-Aldrich), followed by infection with WNVKUN strain MRM61C at a m.o.i. of 3 to 5 as previously described (Westaway et al., 1997b). Infected cells were maintained in...
Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% BSA for 24 h at 37 °C. To assess the effect of IFN on the production of virus particles, tissue culture fluid (tcf) was collected 24 h post-infection (p.i.) and viral titres were analysed by plaque assays. In untreated A549 cells, WNVKUN titre was $5.6 \times 10^6$ p.f.u. ml$^{-1}$ (Fig. 1a). In contrast, WNVKUN virus production was inhibited by 97.7% by pretreatment with 1000 IU IFN-α (1.3 $\times 10^6$ p.f.u. ml$^{-1}$), whereas IFN-γ had a lesser inhibitory effect on the production of virus particles (4.7 $\times 10^5$ p.f.u. ml$^{-1}$ or a 16.1% decrease). Additionally, the effect of IFN on WNVKUN replication was further investigated by analysing the expression of the WNVKUN nonstructural protein NS5 under similar conditions. Western blotting of harvested lysates revealed that the expression of NS5 was severely impaired when infected cells were pretreated with IFN-α, and to a lesser extent after IFN-γ treatment (Fig. 1b). The observed inhibitory effects of IFN-α correlated well with the apparent increased expression of the IFN-induced MxA protein (Fig. 1b). Interestingly, MxA expression was also induced during infection of cells with WNVKUN in the absence of IFN stimulation. These observations are in agreement with previous research indicating increased MxA expression during infection of cells with Dengue virus and WNV (Guo et al., 2003; Warke et al., 2003). These results indicate that the type I IFN-induced antiviral response is effective against WNVKUN replication and virus production, and suggests that the presence of MxA may contribute to this inhibitory effect.

Although IFN can prevent flavivirus infection, it is not fully understood what role IFN-induced proteins like MxA play during this prevention. Recent research has shown that severe acute respiratory syndrome coronavirus (SARS-CoV) and hepatitis C virus (HCV) are not inhibited by MxA in cells constitutively expressing the MxA protein (Spiegel et al., 2004; Frese et al., 2001); however, the clinical outcomes of infection are still influenced by the MxA protein. Polymorphisms in the MxA gene influenced IFN-responsiveness in HCV-infected patients (Suzuki et al., 2004) and were also associated with increased susceptibility to SARS-CoV infection (Spiegel et al., 2004).

In this study, we sought to investigate the role of human MxA during WNVKUN infection. We therefore utilized a Vero cell line stably expressing the human MxA protein (VMxA, clones A3 and A9; Frese et al., 1995), and investigated whether WNVKUN could efficiently replicate in these cells when compared to normal Vero cells. Cells were fixed 24 h post WNVKUN infection in acetone/ethanol (1:1) for 20 min at −20 °C for immunofluorescence (IF). For detection of human MxA, MxA-specific rabbit polyclonal and mouse monoclonal (M143) antibodies were used (Ponten et al., 1997; Flohr et al., 1999). Our initial IF analysis of WNVKUN-infected Vero and VMxA cells at 24 h p.i. revealed efficient staining for the WNVKUN non-structural protein NS3 within both infected Vero and VMxA cells (Fig. 1c). Both staining patterns were consistent with that observed previously for WNVKUN NS3 (Westaway et al., 1997b) and strongly suggested efficient replication and translation of the WNVKUN viral RNA in the VMxA cells. In addition, the comparative staining pattern of MxA between mock- and WNVKUN-infected cells appeared similar (Fig. 1c), suggesting that MxA itself was not redistributed upon infection. Although it appeared that viral replication was unaffected in VMxA cells, any possible impact of MxA on WNVKUN replication was further assessed by infecting VMxA cells with virus-like particles (VLPs) encapsulating a WNVKUN replicon encoding the β-galactosidase reporter gene (Liu et al., 2002). Cell lysates were prepared 48 h p.i. and the β-galactosidase assay was performed according to

**Fig. 1.** Type I IFN inhibits WNVKUN replication and secretion in A549 cells. (a) Plaque assay of collected tcf from WNVKUN-infected A549 cells pretreated for 6 h with 1000 IU IFN-α or 100 ng IFN-γ. (b) Western Blot analysis of lysates prepared from WNVKUN or mock-infected A549 cells pretreated with IFN-α or IFN-γ. Treatment of cells with IFN-α-induced MxA protein expression in both uninfected and WNVKUN-infected cells with a corresponding decrease in WNVKUN NS5 expression. (c) IF of WNVKUN-infected VMxA cells. Subcellular distribution of the WNVKUN NS3 is detected with monospecific antisera and Oregon Green and MxA with anti-MxA antibodies and Texas Red. (d) β-Galactosidase expression assay of lysates collected from Vero and VMxA cells infected with VLPs encoding WNVKUNrep3-β-gal. Plaque assay of tcf harvested from WNVKUN-infected Vero and VMxA cells at 24 h p.i.
the Promega protocol ‘β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer’. β-Galactosidase production was assessed at 405 nm using a plate reader (Murex MRX) and calculated by comparison with a β-galactosidase standard using Dynex Revelation 3.04 software as previously described (Liu et al., 2002). The β-galactosidase assay revealed no significant differences in virus RNA replication between the two cell types (Vero cells: 7.13 milliunits ml\(^{-1}\) and VMxA cells: 6.25 milliunits ml\(^{-1}\), Fig. 1d). Although efficient RNA replication and translation were observed, we further investigated whether MxA could influence the production of secreted WNV\(_{KUN}\) virions. Plaque assay analysis of tcf harvested from WNV\(_{KUN}\)-infected Vero and VMxA cells 24 h p.i. again revealed there was no significant difference in virus titre between cells with and without MxA protein production (1.4 × 10\(^7\) and 1.3 × 10\(^7\), respectively; Fig. 1d). Thus, it appeared that WNV\(_{KUN}\) RNA replication, translation and virus production was unaffected in the presence of the MxA protein.

To investigate whether the ability of WNV\(_{KUN}\) virus to resist inhibition by expression of MxA was due to the interaction of MxA with a virus-encoded antagonist, we compared the intracellular distributions of each of the WNV\(_{KUN}\) proteins in infected VMxA cells with that of the distribution of the expressed MxA protein. A range of mono-specific anti-WNV\(_{KUN}\) monoclonal and polyclonal antibodies that have been described previously (Khromykh et al., 1999; Mackenzie et al., 1998; Westaway et al., 1997a; Mackenzie & Westaway, 2001) were used. Coincident dual-labelling of MxA with a viral protein could strongly suggest interaction at that site. Fig. 2(a) shows the distribution of the WNV\(_{KUN}\) proteins and the MxA protein in WNV\(_{KUN}\)-infected VMxA cells and revealed no obvious colocalization of the WNV\(_{KUN}\) proteins and MxA. To further analyse possible binding between MxA and WNV\(_{KUN}\) proteins, a radio-immunoprecipitation (RIP) assay of WNV\(_{KUN}\)- or mock-infected VMxA cells was performed. Subconfluent VMxA cell monolayers were infected with WNV\(_{KUN}\) at a m.o.i. of 5. At 19 h p.i., cells were incubated in methionine- and cysteine-deficient medium for 1 h. Subsequently, the cells were radiolabelled with 100 μCi ml\(^{-1}\) (3.7 MBq ml\(^{-1}\)) of \([^{35}\text{S}]\)methionine-cysteine (Trans\(^{35}\)S-label; ICN) for 4 h at 37 °C. The cells were harvested in co-immunoprecipitation buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100) containing protease inhibitors (50 mM PMSF and 5 μg ml\(^{-1}\) leupeptin) and incubated on ice for 20 min before clarification by centrifugation at 4 °C. The resulting supernatant was used for RIP with a monoclonal antibody to MxA. Fig. 2(b) indicates no direct interaction between MxA and WNV\(_{KUN}\) proteins since no viral proteins were co-isolated with the MxA antibody. These results suggest that the resistance of WNV\(_{KUN}\) to MxA expression is not mediated by a WNV\(_{KUN}\)-encoded MxA antagonist that was specifically modulating the antiviral properties of MxA via a direct interaction.

Replication of WNV\(_{KUN}\) is associated with virus-induced membrane structures within the cytoplasm of infected cells; these membranes appear as packets of vesicles (VP) associated with the sites of viral RNA synthesis and as convoluted membranes (CM) and paracrystalline arrays (PC) containing the components of the virus-specified protease (Mackenzie et al., 1998, 1999; Westaway et al., 1997b). An alternative explanation for the lack of MxA action against WNV\(_{KUN}\) may reside in the fact that the
replication of flaviviruses within induced membrane structures ‘hides’ viral components such as dsRNA or nucleocapsid from IFN-induced antiviral mediators. In order to investigate this, we analysed the replication of WNVKUN in VMxA cells and Vero cells in the absence of the virus-induced membranes. Brefeldin A (BFA) is a Golgi apparatus-disrupting agent which prevents the development of virus-induced membranes when added before the end of the latent period (Mackenzie et al., 1999), yet appears to still allow WNV KUN RNA replication (Mackenzie & Westaway, 2001). Thus, Vero and VMxA cells were infected with WNVKUNrep3-β-gal VLPs and, at 12 h p.i., BFA (5 μg ml⁻¹) was added to the culture medium for an additional 12 h. The effect of BFA on Golgi apparatus disruption was assessed by IF using Golgi-specific anti-giantin antibodies. In the presence of BFA, the Golgi apparatus was clearly disrupted as giantin was observed to be more diffuse throughout the cell compared to the untreated cells (Fig. 3b). To compare WNVKUNrep3-β-gal replication in MxA protein-expressing cells in the presence and absence of virus-induced membranes, a β-galactosidase assay was performed. In the absence of BFA, Vero and VMxA cells showed comparable β-galactosidase expression (Fig. 3a; 100 % and 99 % relative β-galactosidase expression for Vero and VMxA cells, respectively). However, in the presence of BFA, RNA replication was reduced by 39 % within the VMxA cells compared to BFA-treated Vero cells (100 % and 61 % relative β-galactosidase expression, respectively, \( P < 0.001 \)). These results suggest that the WNVKUN-induced membranes may provide partial protection against the human MxA protein.

Here we showed that the characteristic membrane structures induced during flavivirus infection, which have been previously shown to facilitate flavivirus replication, also provide protection of the viral replication machinery from the antiviral activity of the IFN-stimulated MxA protein. This escape mechanism appears to complement the recently identified roles of the flavivirus non-structural proteins in repression of the host antiviral response.

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**References**


