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Equine MX2 is a restriction factor of equine infectious anemia virus (EIAV)

Kristina Meier[a], Ananda Ayyappan Jaguva Vasudevan[a], Zeli Zhang[a], Ariane Bähr[a], Georg Kochs[b], Dieter Häussinger[a], Carsten Münk[a,*]

[a] Clinic for Gastroenterology, Hepatology, and Infectiology, Medical Faculty, Heinrich-Heine-University, Moorenstr. 5, 40225 Düsseldorf, Germany
[b] Institute of Virology, Medical Center, University of Freiburg, Faculty of Medicine, University of Freiburg, Herman-Herder-Str. 1, 79104 Freiburg, Germany

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ABSTRACT

Human myxovirus resistance protein B (hMXB) is a restriction factor of HIV-1 that also inhibits a variety of retroviruses. However, hMXB is not antiviral against equine infectious anemia virus (EIAV). We show here that equine MX2 (eMX2) potently restricts EIAV *in vitro*. Additionally, eMX2 inhibits HIV-1 and other lentiviruses, including murine leukemia virus. Previously, it was reported that hMXB repression is reduced in hMXB Δ1–25, but not in GTP-binding mutant K131A and GTP-hydrolysis mutant T151A. In contrast to this phenomenon, our study indicates that eMX2 restriction is not diminished in eMX2 Δ1–25, but is in eMX2 K127A and T147A, which correspond to hMXB K131A and T151A, respectively. Thus, eMX2 may inhibit retroviral replication by a novel mechanism that differs from that of hMXB.

1. Introduction

Myxovirus resistance (MX) proteins, of the family of dynamin-like GTPases, are antiviral factors of the innate cell-autonomous immune response, and their expression is tightly regulated by type I (α and β) and type III (λ) interferon stimulus upon viral infection (Holzinger et al., 2007; Mordstein et al., 2008). MX proteins can be found in most vertebrates, with most mammals expressing two different MX proteins that are, in the case of humans referred to as MXA and MXB, but for other mammals commonly referred to as MX1 and MX2, which are encoded by the *MX1* and *MX2* genes, respectively. These two genes arose from ancient gene duplication, and the human MXA protein is more closely related to bovine, canine, equine, porcine and bat MX1 than to the human MXB protein, which in turn shows a high homology with the MX2 proteins of these species (Haller et al., 2007). Rodents are an exception since they lost the ancient *Mx2* gene. The original *Mx1* gene underwent duplication in rodents, resulting in two paralogous gene products, Mx1 and Mx2, that are both closely related to human MXA and not to MXB (Mitchell et al., 2015). Murine Mx1 and its antiviral activity against influenza A virus were first discovered as early as the 1960s (Lindenmann, 1962, 1964). Human MXA has a broad antiviral activity and targets diverse viruses, including orthomyxoviruses, paramyxoviruses, bunyaviruses and rhabdoviruses by different mechanisms such as binding to and trapping the nucleocapsid in the cytoplasm and preventing nuclear import or viral mRNA synthesis, or by the sequestration of viral proteins (Kochs and Haller, 1999; Kochs et al., 2002b; Reichelt et al., 2004; Schwemmle et al., 1995b; Staeheli and Pavlovic, 1991; Xiao et al., 2013; Zhao et al., 1996). The human immunodeficiency virus type 1 (HIV-1) and other retroviruses, on the other hand, were considered to be non-sensitive to MX proteins. MXB was long thought to lack any antiviral activity, but has recently been identified as a restriction factor of HIV-1 and herpesviruses (Crameri et al., 2018; Goujon et al., 2013; Jaguva Vasudevan et al., 2018; Kane et al., 2013; Schilling et al., 2018). Restriction of HIV-1 occurs at a late post-entry step, disturbing the uncoating and nuclear entry process (Fricke et al., 2014; Goujon et al., 2013; Kane et al., 2013; Liu et al., 2013; Matreyek et al., 2014). Human MXB exists in two isoforms that are translated from the same mRNA, but starting from two alternative ATGs generating long or short isoforms that differ by the presence of the first 25 N-terminal amino acids (Melén et al., 1996). Restriction of HIV-1 requires capsid-binding via the N-terminal domain of MXB, which also constitutes a nuclear localization signal (NLS) and thus HIV-1 replication is only restricted by the long isoform (Fricke et al., 2014; Schulte et al., 2015). Mutant analyses showed that apart from the NLS, GTP-binding, but not hydrolysis, is necessary for nuclear localization of human MXB (King et al., 2004; Matreyek et al., 2014). Previous studies suggested that the antiviral activity against HIV-1 is dependent on cyclophilin A (CypA), since HIV-1 capsid mutants that are unable to bind CypA escape restriction and wild-type HIV-1 infection of MXB-expressing cells can be rescued with cyclosporine A (Liu et al., 2013, 2015).

* Correspondence to: Clinic for Gastroenterology, Hepatology, and Infectiology, Medical Faculty, Heinrich-Heine-University Düsseldorf, Building 23.12.U1.82, Moorenstr. 5, 40225 Düsseldorf, Germany.
E-mail address: carsten.muenk@med.uni-duesseldorf.de (C. Münk).

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HIV-1 replication is potently blocked by simian MXB proteins isolated from macaques and African green monkey, whereas, despite having high sequence homology to human and simian MXB, neither canine nor ovine MX2 restricts HIV-1 (Busnadiego et al., 2014). In addition to HIV-1, human MXB has been found to restrict HIV-2 and various simian immunodeficiency viruses (SIVs), but has no or only a very modest effect on equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV) (Busnadiego et al., 2014; Goujon et al., 2013; Kane et al., 2013).

EIAV causes a persistent infection in equids with a primary acute phase that enters a chronic phase, which is associated with recurring cycles of viremia and during which various symptoms, such as fever, anemia, edema, and thrombocytopenia, can occur (Leroux et al., 2004). EIAV is quite unique among lentiviruses in that the chronic phase that typically lasts approximately one year is followed by a lifelong asymptomatic phase, indicating that, in contrast to HIV-1, EIAV can ultimately be controlled by the host immune system and, therefore, it is subject to extensive vaccine studies (Craigo et al., 2007; Leroux et al., 2004). While felines do not express an MXB protein (Busnadiego et al., 2014; Mitchell et al., 2015), databank analyses indicated the existence of a full-length equine *MX2* gene.

We, therefore, decided to examine the antiviral activity of equine MX2 on EIAV infection in comparison to human MXB.

2. Material and methods

2.1. Plasmids

Wild-type human and equine MX2 as well as mutants were expressed by lentiviral vectors pLVX-IRES-Puro (Takara Clontech, Saint-Germain-en-Laye, France). The full-length ORF of hMXB (NM_002463) (Aebi et al., 1989) with an N-terminal FLAG epitope was cloned into the *Spe*I x *Not*I restriction sites using PCR primers forward (fw) 5'- act act agt acc atg gac tac aag gac gac gac gac aaa tct aag gcc cac aag cct tg-3' and reverse (rv) 5'-atg cgg ccg ctc agt gga tct ctt tgc tgg -3', creating pLVX-IRES-Puro-FLAG-hMXB. The full length ORF of eMX2 (XM_005606159) was obtained by RNA extraction from E. Derm cells (ATCC CCL-57) treated with 500 U/ml human IFN β in 6-well dish for 24 h using TRIzol (Life Technologies, Darmstadt, Germany), and cDNA conversion using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Schwerte, Germany). It was then cloned into the *Spe*I x *Not*I restriction sites with an N-terminal FLAG epitope using fw PCR primer 5'- act act agt acc atg gac tac aag gac gac gac gac aaa agt ttc tcc cag caa cgg cc -3' and rv primer 5'- atg cgg ccg ctt aac tgg aga att taa aga -3', creating pLVX-IRES-Puro-FLAG-eMX2. Our cloned sequence is identical to the sequence found in the databank. Mutants and chimeric proteins with an N-terminal FLAG tag were generated via overlapping PCR and cloned into the *Spe*I x *Not*I restriction sites creating pLVX-IRES-Puro-FLAG-hMXBΔNLS (using primer 5' -act act agt acc atg gac tac aag gac gac gac gac aaa aat tcc ttc cag caa cag cc -3' and rv 5'-atg cgg ccg ctc agt gga tct ctt tgc tgg -3'), pLVX-IRES-Puro-FLAG-hMXBK131A (using primers 5'- cgg gga cca gag ctc ggg cgc gag ctc tgt gct gga gg -3' and 5'- cct cca gca cag agc tcg cgc ccg agc tct ggt ccc cg -3'), pLVX-IRES-Puro-FLAG-hMXBT151A (using primers 5'- ccc aga ggc agc gga atc gtg gcc agg tgt ccg ctg gtg ctg -3' and 5'- cag cac cag cgg aca cct ggc cac gat tcc gct gcc tct ggg -3'), pLVX-IRES-Puro-FLAG-eMX2ΔNLS (using primers 5'- act act agt acc atg gac tac aag gac gac gac gac aaa att ccc cca aac ggg cag gt -3' and 5'- atg cgg ccg ctt aac tgg aga att taa aga -3'), pLVX-IRES-Puro-FLAG-eMX2K127A (using primers 5'-gac cag agc tcg ggc gcg agc tct gtg ctg gag -3' and 5'- ctc cag cac aga gct cgc gcc cga gct ctg gtc -3' in addition to the above mentioned primers for wild-type FLAG-eMX2) and pLVX-IRES-Puro-FLAG-eMX2T147A (usingprimers 5'- ggc agc gga atc gta gcc agg tgt ccg ctg gtg -3' and 5'-cac cag cgg aca cct ggc tac gat tcc gct gcc -3' in addition to the above mentioned primers for wild-type FLAG-eMX2) as well as pLVX-IRES-Puro-FLAG-hMXB(NeMX2) (using primers 5'- act act agt acc atg gac tac aag gac gac gac gac aaa agt ttc tcc cag caa cgg cc -3', 5'- ggt gtg gag cag gac ata gcc ctg cca gcc atc gcc gtc atc -3', 5'- gat gac ggc gat ggc tgg cag ggc tat gtc ctg ctc cac acc -3' and 5'- atg cgg ccg ctc agt gga tct ctt tgc tgg -3') and pLVX-IRES-Puro-FLAG-eMX2(NhMXB) (using primers 5'- act act agt acc atg gac tac aag gac gac gac gac aaa tct aag gcc cac aag cct tg -3', 5'- ggt gtg gag cag gac ctg gcc ctg ccc gct atc gcc gtc atc -3', 5'- gat gac ggc gat agc ggg cag ggc cag gtc ctg ctc cac acc -3' and 5'- atg cgg ccg ctt aac tgg aga att taa aga -3').

2.2. Cell lines

HOS (ATCC CRL-1543), HeLa (ATCC CCL-2) HEK293T (ATCC CRL-3216) and E. Derm (ATCC CCL-57) cells were maintained in Dulbecco's modified Eagle's medium (PAN-Biotech, Aidenbach, Germany) supplemented with 10% FBS, 0.29 mg/ml L-glutamine, and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Stable MX protein expressing cell lines were generated by transduction with lentiviral vectors made by transfection of pLVX-IRES-Puro (empty vector) or pLVX-IRES-Puro encoding wild-type hMXB, eMX2 or mutants, together with psPAX2 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, catalogue number 11348), pRSV-Rev (Dull et al., 1998) and pMD.G (Emi et al., 1991). Puromycin resistant cell pools were tested for MX protein expression by immunoblots.

2.3. Cell proliferation analysis

For assessing cell proliferation, cell viability was determined over a period of four days by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay (MTT, Sigma Aldrich, Germany) in quadruplicate, as described before (Jaguva Vasudevan et al., 2018). 10 µl MTT-stock solution (5 mg/ml in PBS) added per well (1000 cells/well seeded and maintained, in 96-well plate) and incubated for 60 min at 37 °C. The formazan product was dissolved by 50 µl DMSO after discarding the medium containing MTT and the absorbance was analyzed spectrophotometrically at 550 nm against the reference wavelength of 750 nm. Wells containing no cells, but medium alone served as blank.

2.4. Generation of viral vectors and infections

Luciferase reporter vectors: Plasmid transfection into HEK293T cells for lentiviral vectors was done with Lipofectamine LTX reagent (Thermo Fisher Scientific). For HIV-1, transfection experiments in 6-well dish consist of 600 ng of the HIV-1 packaging constructs pMDLg/PRRE (Dull et al., 1998), 250 ng of the HIV-1 Rev expression plasmid pRSV-Rev, 600 ng of the HIV-1 reporter vector pSIN.PPT.CMV.Luc.IRES.GFP (Bähr et al., 2016), and 200 ng of the VSV-G expression plasmid pMD.G. For HIV-2, transfection in 6-well dish consists of 425 ng pHIV-2Δ4 (Morris et al., 2004), 125 ng pMD.G, 300 ng HIV-2 Luc SV40 (Kloke et al., 2010), and 300 ng pcDNA3.1 (Thermo Fisher Scientific). $EIAV_{PV}$ luciferase vectors were produced by transfection in 6-well dish with 1 µg pONY3.1, 1 µg pONY8.1luc (Zielonka et al., 2009), and 250 ng pMD.G. $EIAV_{FDDV12}$ reporter viruses were obtained by transfection in 6-well dish with 1 µg VR-Gp2 (Yin et al., 2014), 1 µg pONY8.1luc, and 250 ng pMD.G. FIV reporter vectors were produced by transfection in 6-well dish of 600 ng of pFP93 (Loewen et al., 2003), a gift from Eric M. Poeschla, 600 ng of FIV-luciferase vector pLiNSin (Münk et al., 2008), and 200 ng of pMD.G; pcDNA3.1 was used to fill up to 2.2 µg total DNA. MLV luciferase vectors were produced by transfection in 6-well dish of 1 µg pHIT60 (Bock et al., 2000), which was kindly provided by Jonathan Stoye, 1 µg MP71luc (Jaguva Vasudevan et al., 2017) and 150 ng pMD.G. For SIVcpzPts and SIVcpzPtt transfection was carried out in 24-well dish with 300 ng SIVcpz*Ptt*MB897-NLuc (Zhang et al., 2017), or SIVcpz*Pts*TAN1-NLuc (Zhang et al., 2017) and 50 ng pMD.G. PFV was prepared as previously described (Bähr et al., 2016). HIV-1 and EIAV particles were concentrated by