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The coronavirus nucleocapsid protein is ADP-ribosylated

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ABSTRACT

ADP-ribosylation is a common post-translational modification, although how it modulates RNA virus infection is not well understood. While screening for ADP-ribosylated proteins during coronavirus (CoV) infection, we detected a ~55 kDa ADP-ribosylated protein in mouse hepatitis virus (MHV)-infected cells and in virions, which we identified as the viral nucleocapsid (N) protein. The N proteins of porcine epidemic diarrhea virus (PEDV), severe acute respiratory syndrome (SARS)-CoV and Middle East respiratory syndrome (MERS)-CoV were also ADP-ribosylated. ADP-ribosylation of N protein was also observed in cells exogenously expressing N protein by transduction using Venezuelan equine encephalitis virus replicon particles (VRPs). However, plasmid-derived N protein was not ADP-ribosylated following transient transfection but was ADP-ribosylated after MHV infection, indicating that this modification requires virus infection. In conclusion, we have identified a novel post-translation modification of the CoV N protein that may play a regulatory role for this important structural protein.

1. Introduction

ADP-ribosylation is the covalent attachment of ADP-ribose (ADPr) moieties to a protein, resulting in either mono-ADPr (MAR) or poly-ADPr (PAR). ADP-ribosylation is catalyzed by poly-ADPr polymerases (PARPs), also known as ADP-ribosyltransferases (ARTDs). The PARP family consists of 17 proteins in humans and 16 in mice, which utilize NAD as the ADPr donor [reviewed in (Bock and Chang, 2016)]. Removal of ADPr from proteins (de-ADP-ribosylation) is catalyzed by different cellular proteins including PAR glycohydrolase (PARG) and macrodomain proteins (Bernardi et al., 1997; Jankevicius et al., 2013; Rosenthal et al., 2013; Sharifi et al., 2013). Detection of ADP-ribosylated proteins on a proteomic level is difficult due to the reactive nature and short half-life of the modification in cells (Cervantes-Laurean et al., 1997; Wielckens et al., 1982). Despite this, studies of individual PARPs and ADP-ribosylated proteins have elucidated several physiological roles for ADP-ribosylation, including DNA damage and repair, regulation of RNA transcription, cellular stress response, inflammation, differentiation, and apoptosis (Bock and Chang, 2016).

PARPs are well-established to have both proviral and antiviral properties. PARP1 has been shown to facilitate Epstein-Barr virus replication and latency, simian virus 40 induction of cellular necrosis, and HIV integration (Gordon-Shaag et al., 2003; Ha et al., 2001; Lupey-Green et al., 2017; Tempera et al., 2010). TiPARP has been shown to inhibit interferon (IFN) production by ADP-ribosylation of TBK-1, leading to enhanced replication of several viruses (Yamada et al., 2016). Finally, ADP-ribosylation of the adenovirus core protein has been implicated in aiding viral replication and modulating stability of viral chromatin-like structures (Dery et al., 1986). Other data suggest that PARPs can also be antiviral. Many PARPs are expressed following IFN stimulation and several PARPs show evidence of rapid evolution, suggesting a microbial “arms race” between PARPs and cellular pathogens (Atasheva et al., 2012; Daugherty et al., 2014; MacDonald et al., 2007). For example, PARP7, PARP10, and PARP12 restrict Venezuelan equine encephalitis virus (VEEV) replication and can block cellular translation when overexpressed (Atasheva et al., 2012, 2014). One notable PARP, the Zinc-finger antiviral protein (ZAP) or PARP13, has been demonstrated to inhibit replication of several different viruses, potentially by binding to viral RNA and directing it to be degraded by the RNA exosome (Bick et al., 2003; Gao et al., 2002; Guo et al., 2004, 2007; Liu et al., 2015; Muller et al., 2007; Zhu et al., 2011). PARP13 is enzymatically inactive, and thus its antiviral activity is independent of ADP-ribosylation. In addition, Liu et al. have described a novel mechanism in which an unknown PARP ADP-ribosylates two subunits of the influenza RNA polymerase, allowing subsequent binding to ZAP and degradation of these subunits by the proteasome (Liu et al., 2015).

Three different virus families, Hepeviridae, Togaviridae, and Coronaviridae, encode for a viral macrodomain, which has been shown to de-ADP-ribosylate proteins *in vitro* (Li et al., 2016; Rosenthal et al., 2013). These macrodomains have been proposed to counter the antiviral effects of ADP-ribosylation during infection. Our previous work has focused on the coronavirus (CoVs) macrodomain. CoVs are large, positive-sense, single-stranded RNA viruses which include human pathogens such as the severe acute respiratory syndrome (SARS)-CoV and

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Middle East respiratory syndrome (MERS)-CoV as well as important veterinary pathogens such as bovine CoV and porcine epidemic disease virus (PEDV). All CoVs encode a macrodomain within non-structural protein 3 (nsp3) that can remove both MAR and PAR from proteins (Li et al., 2016). CoVs lacking this enzymatic activity generally replicate normally *in vitro* but are highly attenuated *in vivo* and elicit an enhanced innate immune response (Eriksson et al., 2008; Fehr et al., 2015, 2016; Kuri et al., 2011).

To identify potential targets of the CoV macrodomain, we analyzed infected cells for changes in ADP-ribosylation patterns utilizing antibodies specific for ADPr. We focused on cells infected with a murine CoV, mouse hepatitis virus (MHV). MHV causes acute and chronic encephalomyelitis, hepatitis and gastroenteritis (Bailey et al., 1949). Surprisingly, we found that the CoV nucleocapsid (N) protein was ADP-ribosylated in cells during infection with MHV as well as several other CoVs.

2. Materials and methods

2.1. Cell culture, plasmids and reagents

Delayed brain tumor (DBT) cells, 17Cl-1 cells, Vero cells, and HeLa cells expressing the MHV receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (HeLa-MHVR) were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum as previously described (Zhou and Perlman, 2007). Codon-optimized MHV-A59 N protein was synthesized and cloned directly into pcDNA3 (GenScript). A tagged construct was synthesized by inserting a 3X-FLAG sequence to the C terminus of the N protein using overlapping primers and recombination by In-fusion (Clontech). Control plasmid pcDNA3-GFP was described previously (Fehr et al., 2016).

2.2. Virus Infection

Recombinant mouse hepatitis virus (MHV) strains A59 (Yount et al., 2002) and JHMV (wild-type and N1347A) (Fehr et al., 2015) were propagated on 17Cl-1 cells, and titers were determined on HeLa-MHVR cells. SARS-CoV (MA15) was propagated and titered on Vero E6 cells, and MERS-CoV (EMC12) and PEDV (ISU13-19338E, a gift from Dr. Kyoung-Jin Yoon, Iowa State University) were propagated on Vero-81 cells. For virus infections, 17Cl-1, DBT, Calu-3, Vero E6, or Vero 81 cells were infected with virus at the indicated multiplicity of infection (MOI) and collected at the indicated hours post-infection (hpi). All work with SARS-CoV or MERS-CoV infectious virus was performed in a biosafety level 3 laboratory according to the guidelines set forth by the University of Iowa.

2.3. Proteinase K treatment of virions

DBT cells were infected with MHV-A59 at an MOI of 0.5 PFU/cell, and supernatant was collected and filtered at 12 hpi. The filtrate was subjected to ultracentrifugation at 27,000 rpm for 4 h over a 30% sucrose cushion as described previously. Pellets were resuspended in 100 mM NaCl and 10 mM Tris-Cl (pH 7.2) and treated with or without Proteinase K (New England Biolabs) in the presence or absence of SDS. The reaction was stopped by incubation at 65 °C for 10 min.

2.4. Virus transfection and transduction

Cells were transfected with PolyJet *In Vitro* Transfection Reagent (SignaGen Labs) as per the manufacturer's instructions. 24 h after transfection, cells were either treated with or without 1000 U/ml of IFN-β (PBL) for 24 h or were infected with MHV-A59 at an MOI of 1 PFU/cell for 12 h before collection. VEEV replicon particles (VRPs) encoding either GFP or MERS nucleocapsid protein were created and titered as previously reported (Zhao et al., 2014, 2016). The VRPs were transduced into Vero 81 cells at indicated MOIs and collected at 24 h post-transduction.

2.5. Immunoblotting

Sample buffer containing SDS, β-mercaptoethanol, protease/phosphatase inhibitor cocktails (Roche), PMSF, PARP inhibitor 3-aminobenzamide (3-AB, Tocris Bioscience), PARG inhibitor adenosine 5′-diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD, Calbiochem) and universal nuclease (ThermoFisher Scientific) was used to collect cell lysates. Proteins were resolved on an SDS polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Following binding with a primary antibody, blots were then visualized by using a peroxidase-conjugated secondary antibody (Thermo Fisher Scientific) detected with a chemiluminescent substrate (Thermo Fisher Scientific) or by using an infrared (IR) dye-conjugated secondary antibody detected with a Li-COR Odyssey Imager (Li-COR, Lincoln, NE). IR secondary antibodies of different wavelengths were used to obtain different signals for antibody bound proteins. Images of α-ADPr- or α-N-stained immunoblots were merged using Image Studio software.

Primary antibodies used for immunoblotting and immunoprecipitation included polyclonal (pAb) α-MHV rabbit serum (Perlman et al., 1987), monoclonal (mAb) α-MHV N (Collins et al., 1982) (mAb 5B188.2, a kind gift from Dr. M. Buchmeier, University of California, Irvine), pAb α-SARS-CoV N (Novus Biologicals), pAb α-nsp3 (gift from Mark Denison, Vanderbilt University), mAb α-PEDV N (gift from Dr. Kyoung-Jin Yoon, Iowa State University), pAb α-MERS-CoV N (Zhao et al., 2016), mouse mAb α-ADPr (10 H, Millipore Sigma), rabbit pAb α-ADPr (Trevigen), chicken pAb α-ADPr (Tulip BioLabs Inc.), α-FLAG (Sigma), α-GAPDH (Poly6314, BioLegend), and α-actin (AC15; Abcam, Inc.) antibodies. Secondary antibodies used included horseradish peroxidase-conjugated α-rabbit or α-mouse (Sigma #A0545/A0168) antibodies or IR-conjugated α-rabbit, α-mouse, or α-chicken (Li-COR, #926–68071/926–32210/925–32218) antibodies.

2.6. Immunoprecipitation

DBT cells infected with MHV-A59 at an MOI of 1 PFU/cell were collected at 12 hpi and pelleted by low-speed centrifugation. Cell pellets were lysed with immunoprecipitation (IP) buffer (0.5% NP-40, 300 mM NaCl, 5% glycerol, and 50 mM Tris pH 8.0) containing protease/phosphatase inhibitor cocktails, PMSF, PARP inhibitor 3-AB, PARG inhibitor ADP-HPD, and a universal nuclease for 2 h at 4 °C. Nuclei were pelleted by centrifugation (16,000 g for 15 min at 4 °C). One aliquot of cell lysate was saved as the input control and boiled in SDS sample buffer described above. Protein G magnetic beads were conjugated to α-ADPr or α-N antibodies (described above) as per manufacturer's instructions (ThermoFisher Scientific). Protein G antibody-conjugated were mixed with cell lysates overnight at 4 °C. Beads were washed with PBS-Tween before elution by boiling in SDS sample buffer.

3. Results

3.1. The MHV nucleocapsid protein is ADP-ribosylated in cell culture

To screen for changes in protein ADP-ribosylation during CoV infection, we infected DBT cells, an astrocytoma cell line, with the A59 strain of MHV. Cells were collected throughout the infection, and cell lysates were immunoblotted with a mouse mAb antibody to ADPr (mAb 10 H). The 10 H antibody has been described to bind preferentially to linear 20 + -mers of PAR with no binding activity to DNA, RNA, or adenosine-monophosphate. However, more recent reports have demonstrated that mAb 10 H also binds to auto-MARylated proteins (Eckei et al., 2017; Goenka et al., 2007; Kawamitsu et al., 1984; Kleine et al., 2008). While the ADP-ribosylation status of most proteins did not change over the course of infection, we noted the appearance of a