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The coronavirus nucleocapsid protein is ADP-ribosylated

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1. Introduction

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-translational modification of the CoV N protein that may play a regulatory role for this important structural protein.
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 Vero E6 cells, and MERS-CoV (EMC12) and PEDV (ISU13-19338E, a gift from Dr. M.E. Grunewald et al.) were propagated on 17Cl-1 cells, and titers were determined on HeLa-MHVR 2002) and JHMV (wild-type and N1347A) (Fehr et al., 2015) were propagated on 17Cl-1 cells, and titers were determined on HeLa-MHVR 2002) and JHMV (wild-type and N1347A) (Fehr et al., 2015).

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 titrated as previously reported (Zhao et al., 2014, 2016). The VRPs were transduced into Vero B1 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-amino-benzamide (3-AB, Tocris Bioscience), PARG inhibitor adenosine 5′-di-phosphate (hydroxymethyl)pyrrolidinodiol (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 SB188.2, 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 horse-radish 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 ADP Pr (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 (Eckel 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
A 55 kDa band at 8 h post-infection (hpi) which increased in abundance up to 12 hpi (Fig. 1A). Immunoblotting with two other commercial α-ADPr antibodies raised in two other species also detected a similar protein band (Fig. 1A). A negative control antibody (α-HA) did not bind to this protein, suggesting a specific interaction of the 55 kDa protein with ADPr antibodies. Based on the size and abundance of this protein, we hypothesized that it was the nucleocapsid (N) protein. Staining with monoclonal α-N antibody produced a signal that completely overlapped the signal from the ADPr antibody (Fig. 1B, left). To confirm that this overlap was consistent in other cell lines and MHV strains, we infected 17Cl-1 cells (a fibroblast cell line) with MHV-A59 or DBT cells with MHV-JHM (JHMV), a neurotropic strain of MHV, and immunoblotted with α-MHV serum or α-ADPr antibody. The results showed that in all cases the N protein completely overlapped with the ~55 kDa ADP-ribosylated protein, demonstrating that N protein ADP-ribosylation is not MHV strain or cell type specific (Fig. 1B, middle and right). To confirm the identity of this protein as the N protein, we collected MHV-infected DBT cells at 12 hpi and immunoprecipitated proteins with either monoclonal α-ADPr or α-N antibodies and immunoblotted with the reciprocal antibodies. As expected, this ~55 kDa protein could be stained with the α-ADPr antibody after immunoprecipitation with α-N and stained with α-N after immunoprecipitation with α-ADPr (Fig. 1C), confirming that the N protein is ADP-ribosylated.

3.2. The Nsp3 macrodomain does not de-ADP-ribosylate the N protein

Previous reports have demonstrated that the nsp3 macrodomain removes both mono- and poly-ADPr from auto-ADP-ribosylated substrates in vitro (Fehr et al., 2016; Li et al., 2016). Because the N protein is known to associate with nsp3, we speculated that the nsp3 macrodomain may de-ADP-ribosylate the N protein (Hurst et al., 2013). To test this, we infected DBT cells with MHV-A59 or MHV-JHM encoding either wild-type or a catalytically-deficient (JHMV-N1347A, A59-N1348A) nsp3 macrodomain and immunoblotted for N protein ADP-ribosylation. If the macrodomain was indeed removing the ADPr from the N protein, we would expect to see increased N protein ADP-ribosylation in cells infected with mutant virus compared to wild-type infected cells. However, we found that the level of the ADP-ribosylated N protein was the same in cells infected with either MHV strain (Fig. 2). These findings suggest that the nsp3 macrodomain does not affect N protein ADP-ribosylation under these conditions.

3.3. The Nucleocapsid protein ADP-ribosylation is conserved in α and β-coronaviruses

Next, we tested whether N protein ADP-ribosylation was conserved in CoV genera and lineages other than MHV. We infected Vero cells with PEDV (α-CoV), SARS-CoV (lineage B β-CoV), or MERS-CoV (lineage C β-CoV) and then collected the cells and analyzed whether the N proteins from these viruses were ADP-ribosylated (Fig. 3). In all cases, ADPr antibody staining overlapped with the N protein of each virus,
demonstrating that this modification is conserved across multiple genera and lineages of CoVs.

3.4. ADP-ribosylation of N protein is retained the MHV virion

It has previously been shown that the N protein is phosphorylated and that some phosphorylated residues depend on whether the protein is intracellular or virion-associated (White et al., 2007). Specifically, MHV N protein at amino acid S197 is phosphorylated in infected cells, but this modification is absent on N protein in virions (Wu et al., 2014).

To determine if N protein ADP-ribosylation is maintained within the virion, we purified virions and analyzed whether N protein from the CoV virion was ADP-ribosylated by immunoblotting. To rule out the possibility of detecting any residual N protein from non-virion sources, pelleted virions were treated with proteinase K with or without virion lysis by SDS. Both the N protein as well as the spike (S) protein were detectable with α-MHV serum in both cells and virions (Fig. 4). Although the S protein was not completely eliminated upon proteinase K treatment, the abundance of the S protein was reduced, consistent with the protein being exposed on the surface of the virus. A co-sedimented nonspecific band of ~70 kDa was also degraded by proteinase K treatment. In contrast, N protein, located in the interior of the virion, was protected from proteinase K treatment unless SDS was also added to lyse the viral envelope. Importantly, immunoblotting with α-ADPr antibody demonstrated that the N protein maintained the ADP-ribosylation in virions.

3.5. Plasmid-expressed N protein is only ADP-ribosylated during infection

To determine if N protein expressed in the absence of CoV infection could be ADP-ribosylated in cell culture, we transduced Vero cells with VEEV replicon particles (VRPs) encoding the MERS-CoV N protein or control GFP at different MOIs (Zhao et al., 2016). Transduced cells were collected at 24 hpi, and immunoblotting of cell lysates showed that N protein expressed from an alphavirus replicon was ADP-ribosylated. (Fig. 5A).

Because the VRP platform utilizes a virus infection to express exogenous proteins, we hypothesized that the ADP-ribosylation of the N protein may require a virus infection. To examine this possibility, we transfected DBT cells with a plasmid encoding codon-optimized MHV-N protein or GFP and then tested whether the N protein was ADP-ribosylated. Because several PARPs are IFN-stimulated genes, we also treated cells with or without IFN-β (Atasheva et al., 2012; MacDonald et al., 2007). Importantly, we were unable to detect any ADP-ribosylation of the exogenous transfected N protein (Fig. 5B). In contrast, a positive control of MHV-A59-infected cell lysate, normalized to total N protein, stained with indicated antibodies. The nucleocapsid (N), spike (S), and a nonspecific (ns) proteins are indicated with arrows.
transfected plasmid expressing GFP or N-FLAG into DBT cells and then mock infected or infected cells with MHV-A59. At 12 hpi, we collected cells and immunoblotted with α-ADPr antibody. The N-FLAG protein was detectable with α-ADPr antibody following infection with MHV-A59 but not in mock infected cells (Fig. 5C). This suggests that N protein is only ADP-ribosylated within the context of virus infection.

4. Discussion

The N protein was initially identified as a major structural protein, binding directly to viral RNA, providing stability to the bound RNA, and self-oligomerizing into the virus nucleocapsid. N protein also binds to the viral membrane (M) protein to facilitate genome loading and viral assembly (Narayanan et al., 2000). It plays a prominent role in transcription and replication of the viral genome (Hurst et al., 2010). In fact, the addition of N protein from an exogenous plasmid is often utilized to initiate cellular infection from recombinant CoV cDNA (Yount et al., 2002). The N protein provides additional accessory functions, including the ability to promote cell cycle arrest, inhibit host translation, and block the IFN response during infection [reviewed in (McBride et al., 2014)]. Many of these functions are regulated by post-translational modifications, most notably phosphorylation by host proteins. For example, SARS-CoV N protein phosphorylation modulates N protein oligomerization, translation suppression, and localization to stress granules (Peng et al., 2008). Furthermore, phosphorylation of the MHV N protein is implicated to function as a cellular switch to control transcription of either genomic or subgenomic RNA (Wu et al., 2014).

In this paper, we have identified an additional post-translational modification of the N protein, ADP-ribosylation, which could also play a role in the regulation of N protein functions.

We have found that the N protein of multiple CoVs was APD-ribosylated in vitro, using multiple antibodies to ADPr (Figs. 1 and 3). The 10 H α-ADPr antibody primarily used in this study binds preferentially to PAR but can be used to detect MARylated proteins as well (Eckei et al., 2017; Goenka et al., 2007; Kawamitsu et al., 1984; Kleine et al., 2008). Because the N protein band stained with either α-ADPr or α-N antibodies did not appear as a smear, which is seen with long-chain PARylated proteins, it is likely that the N protein is either MARylated or PARylated with only a few monomers of ADPr. This is further supported by the fact that ADP-ribosylation did not alter migration of the N protein to a detectable level (Fig. 5B and C). Furthermore, our data indicate that the N protein ADP-ribosylation was detectable during infection (Fig. 1, Fig. 5A, Fig. 5C) but not following transfection alone (Fig. 5B). This could be due to a number of factors including, but not limited to, virus infection-dependent expression of an ADP-ribosylating enzyme or the localization of the N protein to a distinct cellular compartment during infection. N protein in infected cells has been shown to localize both in the cytoplasm as well as the nucleus, and the SARS N protein has been shown to localize to stress granules under stress conditions (Hiscox et al., 2001; Peng et al., 2008). Several PARPs are known to co-localize with stress granules, which could potential sites of N protein modification (Leung et al., 2011). Future experiments are required to
parse out the number of ADP–ribose moieties from the N protein to identify the amino acids that are ADP–ribosylated and to demonstrate the effect of this modification on virus infection.

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Conflicts of interest

None.

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