Minute virus of mice small non-structural protein NS2 localizes within, but is not required for the formation of, Smn-associated autonomous parvovirus-associated replication bodies

Philip J. Young,1† Ann Newman,2† Klaus T. Jensen,3 Lisa R. Burger,2 David J. Pintel2 and Christian L. Lorson1

1Department of Veterinary Pathobiology, Life Sciences Center 471G, University of Missouri, Columbia, MO 65211-7310, USA
2Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO 65212, USA
3Novozymes, 1AS31, Novo Alle, DK-2880 Bagsværd, Denmark

The non-structural proteins NS1 and NS2 of the parvovirus minute virus of mice (MVM) are required for efficient virus replication. It has previously been shown that NS1 and NS2 interact and colocalize with the survival motor neuron (Smn) gene product in novel nuclear structures that are formed late in infection, termed Smn-associated APAR (autonomous parvovirus-associated replication) bodies (SAABs). It is not clear what molecular viral intermediate(s) contribute to SAAB formation. The current results address the role of NS2 in SAAB formation. In highly synchronized wild-type MVM infection of murine A92L cells, NS2 colocalizes with Smn and other SAAB constituents. An MVM mutant that does not produce NS2 still generates SAABs, albeit with a temporal delay. The lag in SAAB formation seen in the absence of NS2 is probably related to the temporal delay in virus replication, suggesting that, whilst NS2 is required for efficient viral infection, it is dispensable for SAAB formation.

Minute virus of mice (MVM) is an autonomous parvovirus consisting of a single-stranded DNA (ssDNA) genome of approximately 5 kb in length (Cotmore & Tattersall, 1986, 1987) that contains two overlapping transcription units under the control of two viral promoters, P4 and P38 (Ahn et al., 1989, 1992; Deleu et al., 1999; Lorson & Pintel, 1997; Lorson et al., 1996). The P4 promoter drives expression of the viral non-structural proteins NS1 and NS2, whilst P38-derived transcripts encode the viral structural proteins VP1 and VP2 (Ahn et al., 1989, 1992; Deleu et al., 1999; Lorson & Pintel, 1997; Lorson et al., 1996). NS2 is a 25 kDa protein found in both the cytoplasm and nucleus of MVM-infected cells (Cotmore & Tattersall, 1990). Although the role of NS2 during infection has been unclear, recent molecular interactions between two cellular proteins, 14-3-3 and Crm1, have shed some light upon potential functions (Bodendorf et al., 1999; Brockhaus et al., 1996; Eichwald et al., 2002; Miller & Pintel, 2002), including a role for NS2 in nuclear egress of MVM particles (Bodendorf et al., 1999; Brockhaus et al., 1996; Eichwald et al., 2002; Miller & Pintel, 2002). In non-permissive cells, the loss of the NS2–Crm1 interaction leads to reduced levels of viral genomic ssDNA and the accumulation of viral capsids in the nucleus (Miller & Pintel, 2002). The role of the NS2–14-3-3 interaction during infection, however, remains unclear. NS2-null mutants have been shown to accumulate partially assembled capsids in non-permissive cells (Cotmore et al., 1997) and these mutants also show severe defects in virus replication that cannot be attributed solely to the lack of assembled capsids (Cotmore et al., 1997; Naeger et al., 1990).

The eukaryotic nucleus is highly organized into numerous distinct, yet highly dynamic, subnuclear structures (Lamond & Carmo-Fonseca, 1993; Lamond & Sleeman, 2003; Lamond & Spector, 2003; Matera, 2003; Platani & Lamond, 2004). A novel subnuclear structure has been identified following infection by the two highly related parvoviruses MVM and H-1 (Bashir et al., 2000; Cziepluch et al., 2000). These structures, termed autonomous parvovirus-associated replication (APAR) bodies, were identified at early time points during infection, however, they are distinct from several of the classically described nuclear bodies, including Cajal bodies, PML bodies (PODs) and interchromatin granules (ICGs) (Bashir et al., 2001; Cziepluch et al., 2000). At later time points during infection, however, a dramatic nuclear reorganization occurs, in which formerly distinct nuclear structures merge into massive structures.
that contain NS1 (Young et al., 2002a, b). These structures are probably active sites of virus production (Young et al., 2002a), but their role during the infection process is not yet known. In addition, the survival motor neuron (Smn) gene product is present in these large structures (hence the term Smn-associated APAR bodies or SAABs). The Smn protein is involved in a variety of cellular activities, including small nuclear ribonucleoprotein biogenesis and functioning as an RNA chaperone (Gubitz et al., 2004; Paushkin et al., 2002). Smn is the first cellular factor identified that interacts with NS1 and NS2 in vitro and in vivo during infection (Young et al., 2002a, b).

We recently reported a direct interaction between Smn and NS2, and our initial observations indicated that the two proteins colocalized within SAABs during MVM infection (Cotmore & Tattersall, 1986, 1987; Young et al., 2002a, b). To characterize the nature of the NS2-positive subnuclear structures formed during infection more extensively, we performed confocal immunofluorescence assays (Young et al., 2002a, b) on highly synchronized A92L cells infected with wild-type MVM (Schoborg & Pintel, 1991; Young et al., 2002a). NS2 localization was monitored at 0, 10, 15, 20 and 30 h post-release and cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to identify nuclei. For all immunofluorescence experiments, parallel reactions were performed with primary antibody or secondary antibody alone to confirm the specificity of the staining (data not shown). At early time points, NS2 was predominantly cytoplasmic, although small nuclear foci were also detected, consistent with the induction of APAR bodies following wild-type MVM infection at early time points (Fig. 1a). At later time points, although still significantly cytoplasmic, NS2 became enriched in the nucleus into subnuclear structures that increased in number and size as the infection proceeded (Fig. 1a). The appearance and temporal formation of the NS2-positive nuclear bodies was consistent with our previous analysis of NS1 and SAABs (Young et al., 2002a, b).

To determine conclusively whether the NS2-positive nuclear structures were similar to previously characterized SAABs and to determine the timing of their appearance, stainings were performed to detect the following SAAB constituents: NS1 and SR proteins, a class of splicing regulatory factors (Fig. 1b, c). As expected, 0 h post-release, NS1 was undetectable and SR proteins were localized within ICGs along the nuclear periphery (Fig. 1b, c). At later time points, SR proteins, as well as NS1 and NS2, concentrated into increasingly larger nuclear foci, establishing the NS2-positive nuclear foci as the previously described SAABs. SR proteins remained within ICGs along the nuclear periphery at all time points in uninfected synchronized cells (data not shown). Previously characterized SAAB components, including Smn and p80 coilin, were also identified within the NS2-positive nuclear foci, further validating these foci as SAABs (data not shown).

Only a limited number of cellular proteins have been shown to interact with the non-structural proteins of MVM. To determine the cellular distribution of a previously described NS2-interacting protein, Crm1 (Bodendorf et al., 1999; Brockhaus et al., 1996), during wild-type MVM infection, similar confocal analyses were performed on highly synchronized MVM infected A92L cells. Typically, Crm1 is enriched at the nuclear periphery, consistent with its role as a nuclear/cytoplasmic transport factor (Fornerod et al., 1997). In highly synchronized A92L cells at 0 h post-release, Crm1 was enriched at the nuclear periphery (Fig. 1d), similar to previous descriptions (Brunet et al., 2002; Fornerod et al., 1997). However, in contrast to non-synchronized human cells (Brunet et al., 2002; Fornerod et al., 1997), the localization of Crm1 in highly synchronized A92L cells also appears to be localized diffusely throughout the cytoplasm (Fig. 1d). NS2 and Crm1 remained localized diffusely in the cytoplasm up to 15 h post-release and, at this point, the accumulation of Crm1 at the nuclear periphery was reduced compared to the 0 h time point. However, at time points beyond 15 h post-release, Crm1 became enriched in SAABs (Fig. 1d). At these later time points, Crm1 colocalized with the majority of NS2 within SAABs, demonstrating that these NS2-interacting proteins are also SAAB components. 14-3-3, an additional NS2-interacting factor, was examined similarly and was shown

Fig. 1. NS2, NS1 and SR splicing factors colocalize in SAABs in MVM-infected A92L cells. (a) Subcellular localization of NS2 at various time points post-release. NS2 (anti-NS2, M55 rabbit polyclonal) [primary, rabbit anti-NS2; secondary, fluorescein isothiocyanate (FITC)/green] is indicated at 0, 10, 15, 20 and 30 h post-release. Synchronized infections were obtained by performing an isoleucine/aphidicolin double block on A92L cells prior to infection. DAPI was used to counterstain nuclei. (b) NS1 and NS2 colocalize in SAABs. NS1 (anti-NS1, 2C9 mouse monoclonal) [primary, mouse monoclonal antibody; secondary, tetramethylrhodamine isothiocyanate (TRITC)/red] and NS2 (primary, rabbit anti-NS2; secondary, FITC/green) are indicated at 0, 15, 20 and 30 h post-release. (c) NS2 and SR splicing factors colocalize in SAABs. NS2 (primary, rabbit anti-NS2; secondary, FITC/green) and SR proteins (anti-SR, 1H4 mouse monoclonal; Zymed Laboratories) (primary, mouse monoclonal; secondary, TRITC/red) are indicated at 0, 15, 20 and 30 h post-release. (d) Crm1, an NS2-interacting protein, colocalizes with NS2 in SAABs. NS2 (primary; rabbit anti-NS2; secondary; TRITC/red) and Crm1 (anti-Crm1: C-20 goat polyclonal; Santa Cruz Biotechnology) (primary, mouse monoclonal; secondary, FITC/green) are indicated at 0, 15, 20 and 30 h post-release. A non-specific staining structure can also be observed at the lower right-hand corner of the 15 h panels. (e) Actin does not colocalize in SAABs. Actin (anti-actin, 10-33 rabbit polyclonal; Sigma) (primary, mouse monoclonal; secondary, TRITC/red) and NS1 (primary, rabbit anti-NS1; secondary, FITC/green) are indicated in infected and uninfected cells 30 h post-release. Bars, approximately 30 μm.
MVM NS2 subcellular localization

(a) NS2  DAPI  Overlay
0 h
10 h
15 h
20 h
30 h

(b) NS1  NS2  Merge
0 h
15 h
20 h
30 h

(c) NS2  SR  Merge
0 h
15 h
20 h
30 h

(d) Crm1  NS2  Merge

(e) Actin  NS1  Merge
30 h infected
30 h uninfected
to redistribute to SAABs after 15 h time points (data not shown). Additional experiments were performed with anti-NS1 and anti-actin antibodies (Fig. 1e). As expected, actin exhibited a typical cytoskeletal-staining pattern and did not localize within the NS1-staining SAABs at 30 h post-infection, demonstrating that specific cellular components are present in SAABs (Fig. 1e).

It is unclear which aspect of the viral infection process induces parvovirus-associated nuclear-body formation (e.g. APAR bodies and SAABs). Whilst NS1 is responsible for the majority of MVM-induced cytotoxicity (Anouja et al., 1997; Corbau et al., 2000), ectopic expression of NS1 and/or NS2 (data not shown) is not sufficient to induce SAAB formation. The following experiments were designed to determine whether NS2 was necessary for SAAB formation in the context of a viral infection. A92L cells were infected at relatively high m.o.i. by the mutant MVM NS2:1989; a virus that does not express NS2 due to a splice-site mutation that prevents the production of the NS2-encoding R2 mRNA (Naeger et al., 1990). At 15 h post-release, little change is observed in the typical subcellular distributions of a number of SAAB factors, including Smn (Fig. 2a), Sm core proteins (Fig. 2b), p80 coilin, SR proteins and PML (data not shown). At this time, APAR bodies are detectable, as evidenced by the majority of NS1-positive nuclear foci clearly being separate from the Smn-positive nuclear gems (Fig. 2a). However, at 30 h post-release, these formerly distinct nuclear components were beginning to merge, consistent with the formation of SAABs (Fig. 2a, b). The number and size of these structures, however, were decreased compared to a wild-type MVM infection at the same time point post-release. SAAB formation has clearly been initiated at 30 h post-release, albeit at a slower rate. In parallel experiments, similar infections were allowed to proceed to 42 h post-release and, at this time point, SAAB formation and the nuclear organization of infected cells closely resembled those of a wild-type MVM infection at 25–30 h post-release (data not shown). Taken together, these results demonstrate that NS2 is not essential for SAAB formation. MVM mutant viruses encoding subtle NS2 mutants that specifically disrupt the interactions with the cellular factors Crm1 and 14-3-3 did not alter the nature or kinetics of SAAB formation (data not shown). These results are consistent with the notion that, whilst NS2 is required for efficient replication, it is not essential for SAAB formation.

Whilst not fully permissive, human NB324K cells can support the replication of MVM NS2-null mutants. MVM NS2:1989 virus was isolated and used to infect NB324K cells as described for wild-type MVM infections of A92L cells. Infection with the MVM NS2:1989 virus induced SAAB formation in NB324K cells, although their appearance was modestly delayed (Fig. 3a, c). This delay, however, was not as pronounced as that seen in MVM NS2:1989-infected non-permissive A92L cells (Fig. 2). No temporal or qualitative defects in APAR body or SAAB formation were detected in NB324K cells infected with wild-type MVM (Fig. 3b, d).

NS2 expression is not essential for SAAB formation, as evidenced by the formation of SAABs in the absence of NS2, although the kinetics of SAAB formation in the absence of NS2 is delayed in A92L cells. Delayed SAAB formation following MVM NS2:1989 infection is probably related to the decreased efficiency in virus replication and infection (Naeger et al., 1990), as the molecular signal(s) that

---

Fig. 2. NS2 is not essential for SAAB formation. (a) NS1 (primary, rabbit anti-NS1; secondary, FITC/green) and Smn (primary, mouse monoclonal; secondary, TRITC/red) are indicated 0, 15 and 30 h post-infection. (b) NS1 (anti-NS1, M50 rabbit polyclonal) (primary, rabbit anti-NS1; secondary, FITC/green) and Sm core proteins (anti-Sm, Y12 mouse monoclonal; Neomarkers) (primary, mouse monoclonal; secondary, TRITC/red) are indicated 0 and 30 h post-infection. Bars, approximately 30 μm.
initiates SAAB formation is still produced. It is of specific interest to determine the detailed kinetic correlation between virus replication and the formation of viral replicative intermediates relative to the MVM-induced nuclear reorganization.

SAAB formation does not seem to be a general cellular response to viral infection, as other viral infections do not induce SAAB formation. Nuclear reorganization, however, is a well-characterized hallmark of a number of pathogens, such as adenovirus and herpesvirus. Whilst adenovirus infection results in the formation of virus replication centres, the molecular constituents are distinct from SAABs (data not shown).

This work also reports an analysis of NS2 subcellular distribution during a highly synchronized MVM infection. Our results are consistent with the previously described ability of NS2 to shuttle between the cytoplasm and the nucleus (Bodendorf et al., 1999; Cotmore & Tattersall, 1990). We find that, early in infection, NS2 is predominantly cytoplasmic and becomes more nuclear at later time points in infection. However, it is unclear whether hyper- or hypophosphorylated NS2 accumulates in SAABs. NS2 redistribution could result from altered NS2 phosphorylation levels or from a global dysregulation of nuclear integrity.

It has become increasingly clear that subnuclear compartments are not merely storage sites, but serve critical cellular functions throughout the cell cycle (Matera & Frey, 1998; Matera, 1999; Ogg & Lamond, 2002). Whether these MVM-specific subnuclear structures represent a viral-induced reorganization that benefits the viral life cycle or whether SAABs represent a cellular defence mechanism in response to a viral infection is a critical question that is currently being investigated. Whilst the majority of the cytotoxic effects of an MVM infection have been attributed to NS1, it is interesting that ectopically expressed NS1 (and/or NS2) cannot induce SAAB formation (Young et al., 2002a; data not shown), suggesting that these structures are not formed in response to these apoptotic or cytotoxic stimuli. Determining the nature of APAR bodies and SAABs and the roles that they play during the parvovirus life cycle will provide insight into the viral life cycle, as well as the interface between host and pathogen.

Acknowledgements

We thank Glenn Morris, Angus Lamond and Colin Parish for antibodies and Constance Chamberlain for technical assistance. K. T. J. was supported by a post-doctoral fellowship from the University of Missouri Life Science Mission Enhancement Program. Funding for these studies was provided by Families of SMA, Andrew’s Buddies (C. L. L., P. J. Y.) and the National Institutes of Health (C. L. L., RO1 NS41584; D. J. P., RO1 AI21302 and RO1 AI46458).

References


Miller, C. L. & Pintel, D. J. (2002). Interaction between parovirus NS2 protein and nuclear export factor Crm1 is important for viral egress from the nucleus of murine cells. *J Virol* 76, 3257–3266.


