Virus Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The role of viral genomic RNA and nucleocapsid protein in the autophagic clearance of hantavirus glycoprotein Gn

Safder S. Ganaie, Mohammad A. Mir*

Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160, USA

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Hantavirus
Glycoprotein
Autophagy
Negative strand RNA virus
Bunyaviridae

ABSTRACT

Hantaviruses have tri-segmented negative sense RNA genome. The viral M-segment RNA encodes a glycoprotein precursor (GPC), which is cleaved into two glycoprotein molecules Gn and Gc that form spikes on the viral envelope. We previously reported that Gn is degraded shortly after synthesis by the host autophagy machinery. However, Gn being an important integral component of the virion, must escape degradation during the packaging and assembly stage of virus replication cycle. The mechanism regulating the intrinsic steady-state levels of Gn during the course of virus replication cycle is not clear. We transfected cells with plasmids expressing viral S-segment RNA, nucleocapsid protein and glycoproteins Gn and Gc and monitored their expression levels over time. These studies revealed that accumulation of nucleocapsid protein, glycoprotein Gc and viral S-segment RNA helped to stabilize Gn. These observations suggest that initiation of virus assembly may help Gn to escape autophagic degradation by yet unknown mechanism.

1. Introduction

Autophagy, originally considered as cellular response to starvation, is an intercellular mechanism required for both the maintenance of cellular homeostasis and generation of pools of energy under stress conditions for the proper functioning of essential cellular process until the normal growth conditions resume (Kudchodkar and Levine, 2009). Autophagy is a cytoplasmic quality control mechanism in which cytosolic components, such as defective or surplus organelles and aggregated proteins are sequestered into double membrane structures, referred as autophagosomes (Deretic, 2010a,b). The autophagosomes serve as vehicles for the selective transport of sequestered cytoplasmic cargo to lysosomes for degradation and clearance. Perturbations to this crucial mechanism result in variety of disease conditions, ranging from infectious diseases to cancer (Levine and Kroemer, 2008). Autophagy begins with the formation of isolated membranes that eventually elongate in size and enclose the cytoplasmic cargo, destined for lysosomal degradation. This multistep process involves an array of genes and is associated with complex membrane dynamics (Levine et al., 2011). While maintaining the cellular homeostasis, this mechanism protects host cells from invading pathogens, including bacteria and viruses. Enforced expression of Beclin 1, an autophagy gene involved in early stages of autophagy induction, reduces viral titers and protects mice against Sindbis virus encephalitis (Liang et al., 1998). Recently, P62, an autophagy adaptor protein has been reported to interact with Sindbis virus nucleocapsid protein and target the viral nucleocapsids to autophagosomes for degradation (Orvedahl et al., 2010). Plants and insects rely on innate and intrinsic immune mechanisms for protection against invading pathogens. Recent studies have suggested that autophagy likely increases the degradation of tobacco mosaic virus (TMV) in infected plant cells (Kudchodkar and Levine, 2009; Liu et al., 2005). Similar to plants, autophagy has been reported to protect *Drosophila* from vesicular stomatitis virus (VSV) infection (Shelly et al., 2009).

Although autophagy is an innate cellular mechanism protecting cells from the attack of invading pathogens such as viruses which, in turn, have evolved strategies to fight back this cellular response for survival and persistence. The three herpesvirus subfamilies (α, β and γ) and lentiviruses either encode proteins with autophagy inhibitory potential or induce cellular signals to inhibit autophagy. For example, α-herpesvirus HSV-1-encoded ICP34.5 protein antagonizes autophagy by direct binding to Beclin 1 (Orvedahl et al., 2007). All γ-herpesviruses encode at least one or two homologues of Bcl2 to counter the clearance of virus-infected cells by the host surveillance machineries (Ku et al., 2008). At least two of these virus-encoded homologues, KSHV vBcl2 and murine γ-HV68 M1, have been reported to directly bind to Beclin 1 for the inhibition of autophagy (Ku et al., 2008; Kudchodkar and Levine, 2009).

Interestingly a growing list of viruses seem to induce autophagy or autophagosome like structures in virus infected cells during the

* Corresponding author at: 402 Orr Major, 3901 Rainbow Blvd., Kansas City, KS 66103, USA. Tel.: +1 913 588 5556; fax: +1 913 588 7295.
E-mail address: mmir@kumc.edu (M.A. Mir).

0168-1702/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.12.034

course of infection (Kudchodkar and Levine, 2009). It is likely that induction of autophagy by these viruses facilitates virus replication and pathogenesis in infected hosts. Epsetin–Barr virus encoded latent membrane protein 1 (LMP1) has been reported to induce autophagy to regulate its own expression levels in cells (Ku et al., 2008). Elevated levels of this protein inhibit mRNA translation and perturb cellular homeostasis (Ku et al., 2008). A number of RNA viruses, such as influenza A, Dengue and hepatitis C virus have been reported to induce autophagy like events in infected cells and require autophagosome like structures for efficient replication. Pharmacological or genetic interventions to inhibit autophagy have resulted in decreased yields of these viruses in infected cells (Ait-Goughoulte et al., 2008; Ku et al., 2008; Zhou et al., 2009). However, further investigations are required to delineate precisely the roles of autophagy proteins, double membrane vesicles and classical autophagic pathways in the virus infectious cycle.

Hantaviruses are negative strand RNA viruses and members of the *Bunyaviridae* family. Pathogenic hantaviruses including Sin Nombre virus (SNV) use avß3 integrins on endothelial cells for entry (Gavrilovskaya et al., 1998). These category A viruses cause serious illness when transmitted to humans through aerosolized excreta of infected rodent hosts (Schmaljohn and Hooper, 2001). Two well characterized diseases caused by hantavirus infection are the hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), having mortalities of 15% and 50% respectively (Schmaljohn, 1996; Schmaljohn and Hooper, 2001). The organs that suffer the pathogenic consequences during HFRS and HCPS are kidneys and lungs, respectively. Although HFRS and HCPS differ in the final organ depicting the diseased pathology, both the diseases primarily cause vascular leakage that leads to shock and hypotension (Macneil et al., 2011). Recently, factor XII-dependent increased activation of the Kallikrein–Kinin System has been proposed to induce endothelial cell permeability during hantavirus infection (Taylor et al., 2013). Annually, 150,000–200,000 hantavirus infections are reported worldwide (Jonsson et al., 2013). The three hantaviral genomic RNA segments S, M and L encode nucleocapsid protein (N), glycoprotein precursor (GPC) and viral RNA dependent RNA polymerase (RdRp), respectively. The GPC is post-translationally cleaved at a conserved WASA-motif, generating an N-terminal fragment (Gn) and a C-terminal fragment (Gc). Both Gn and Gc are packaged into viral envelop during virus assembly. The 142 amino acid long C-terminal tail domain of Gn carries out multiple functions for the successful establishment of viral infection in infected cells. The interaction between Gn cytoplasmic tail domain and the N protein has been proposed to play a role in virus assembly (Hepojoki et al., 2013b; Wang et al., 2013).

We have recently found that Gn expression in cells by either plasmid transfection or virus infection induces autophagy in cells. Interestingly, the activated autophagy response targets Gn for degradation by autophagy–lysosome pathway. The autophagic clearance of Gn was found to be required for efficient hantavirus replication in cells. Here, we demonstrate that co-expression of viral genomic RNA and hantavirus nucleocapsid protein in cells protect Gn from autophagic degradation. Although the virus infected cells are continuously undergoing degradative autophagy, it is still a mystery that how the elevated levels of nucleocapsid protein and viral genomic RNA facilitate the escape of Gn from autophagic degradation.

2. Results and discussion

To better understand the role of viral genomic RNA and nucleocapsid protein on the autophagic degradation of SNV Gn protein, we first provide an overview of our previously published results, followed by new data demonstrating that accumulation of viral genomic RNA and nucleocapsid protein in cells protect the degradation of Gn by the host autophagy machinery. To monitor the expression of SNV glycoprotein precursor (GPC) in cells, we previously fused the green fluorescence protein (GFP) at either N or C-terminus of the GPC and expressed the fusion protein in HeLa cells (Hussein et al., 2012). The GFP expression was monitored by FACS analysis at increasing time intervals. Since both Gn and Gc are expressed from a single mRNA, it was expected that both proteins would be equally expressed in cells. Interestingly, we observed a negligible GFP signal in cells expressing GPC precursor containing an N-terminal GFP tag (Hussein et al., 2012). In contrast, a significantly stronger GFP signal was observed in cells expressing GPC precursor containing a C-terminal GFP tag. To rule out the possibility that GFP fusion at the N-terminus of GPC precursor perturbed the GFP signal, we cloned Gn and Gc separately and expressed them in HeLa cells as GFP fusion proteins. Again, we observed a strong GFP signal in HeLa cells expressing Gc-GFP fusion protein in comparison to cells expressing Gn-GFP fusion protein (Hussein et al., 2012). These observations suggested that Gn was likely degraded in cells, which was finally verified by western blot analysis using either anti-Gn or anti-GFP antibodies (Hussein et al., 2012).

It has been previously reported that the cytoplasmic tail domain of Gn from New York hantavirus, expressed from a plasmid, is ubiquitinated and degraded by the proteasomal machinery in cells (Geimonen et al., 2003). Based on this information, we asked whether the cytoplasmic tail domain of SNV Gn is mediating the degradation of the entire Gn molecule in cells. Interestingly, we found that Gn mutant lacking the C-terminal tail domain was still degraded in cells similar to wild type Gn (Hussein et al., 2012). To determine whether the wild type Gn and Gn mutant lacking the tail domain were degraded by the proteasome machinery, we added a proteasomal inhibitor (MG132) to cells four hours post-transfection and monitored the GFP signal over time. These studies revealed that degradation of both wild type and mutant Gn was not carried out by the proteasome, suggesting that signal required for the degradation of Gn is not present in its tail domain (Hussein et al., 2012). In comparison, the MG132 treatment revealed that the cytoplasmic tail domain expressed separately from a plasmid was degraded by the proteasome machinery, consistent with similar observations from other investigators (Geimonen et al., 2003). However, a treatment with the autophagy inhibitors 3-methyladenine (3MA), LY-294002 (LY) or Wortmanin (Wort) showed that unlike the tail domain both wild type and mutant Gn were degraded by the host autophagy machinery (Hussein et al., 2012). To rule out the possibility that GFP fusion at the N-terminus of Gn did not lead to the degradation of Gn in cells, we monitored the expression of both wild type Gn and GFP-Gn fusion proteins by western blot analysis using either anti GFP or anti-Gn antibody. This analysis revealed that both wild type Gn and GFP-Gn fusion proteins were degraded in cells to an undetectable level (Hussein et al., 2012). However, addition of 3MA equally rescued both wild type Gn and GFP-Gn fusion proteins, suggesting that GFP tag at the N-terminus of Gn did not play any role in the degradation of Gn. The western blot analysis using anti-GFP antibody revealed that GFP tag was not cleaved from the GFP-Gn fusion protein by the N-terminal signal peptide of Gn (Hussein et al., 2012). Our published results demonstrate that that addition of 3MA did not play any inhibitory role in the cleavage of N-terminal GFP tag. This is supported by the fact that no enhancement of GFP signal was observed in cells lacking 3MA. The cleaved GFP from the N-terminus of Gn in absence of 3MA would have easily been detected by either western blot analysis or fluorescence microscopy or FACS analysis, which was not the case. These observations suggest that the N-terminal signal peptide of wild type Gn is likely not cleaved after its translocation through the ER. Interestingly, it has been previously reported that N-terminal signal peptide of Hantaan virus Gn

Please cite this article in press as: Ganaie, S.S., Mir, M.A., The role of viral genomic RNA and nucleocapsid protein in the autophagic clearance of hantavirus glycoprotein Gn. Virus Res. (2014), http://dx.doi.org/10.1016/j.virusres.2013.12.034