Degradation and aggresome formation of the Gn tail of the apathogenic Tula hantavirus

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The cytoplasmic tails of envelope glycoprotein Gn of pathogenic hantaviruses but not of the apathogenic Prospect Hill virus (PHV) were recently reported to be proteasomally degraded in simian COS7 cells. Here, we show that the cytoplasmic tails of the glycoproteins of the apathogenic hantaviruses Tula virus (TULV) and PHV are also degraded through the ubiquitin-proteasome pathway, both in human HEK-293 and in simian Vero E6 cells. TULV Gn tails formed aggresomes in cells with proteasomal inhibitors. We conclude that degradation upon aggregation of Gn tails, which may represent a general cellular response to misfolded protein used by hantaviruses to control maturation of virions, is unrelated to pathogenicity.

INTRODUCTION

Hantaviruses, members of the family Bunyaviridae, are known to cause two severe human diseases: hemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome (HCPS) (Lee & van der Groen, 1989; Peters & Khan, 2002; Vapalahti et al., 2003). Hantaviruses have a tri-segmented negative-stranded RNA genome. The S segment encodes the nucleocapsid protein, M segment two glycoproteins, Gn and Gc, and the L segment encodes the RNA polymerase (Plyusnin et al., 1996). Gn and Gc are co-translationally cleaved from a precursor and targeted to the cis-Golgi compartment (Shi & Elliott, 2002; Spiropoulou et al., 2003). The Gn glycoprotein consists of an external domain, a transmembrane domain and a C-terminal cytoplasmic domain (Spiropoulou, 2001).

Recently, several reports describing properties of the hantavirus Gn cytoplasmic tail (Gn tail) have been published. The Gn tail of HCPS-causing hantaviruses contain immunoreceptor tyrosine-based activation motifs, which direct receptor signalling within immune and endothelial cells (Geimonen et al., 2003). Expression of the New York-1 (NY-1) hantavirus Gn tail inhibited beta interferon promoter activity induced by RIG-I and TBK-1 in a dose-dependent manner (Alff et al., 2006). It was also reported that the Gn tails of pathogenic (NY-1, Andes and Hantaan) but not of apathogenic (Prospect Hill virus; PHV) hantaviruses were proteasomally degraded in COS7 cells (Geimonen et al., 2003; Sen et al., 2007).

We observed that the Gn tails of two apathogenic hantaviruses [Tula virus (TULV) and PHV], both carried by members of the genus Microtus (Vapalahti et al., 1996), are degraded through the ubiquitin-proteasome pathway both in human epithelial kidney (HEK-293) and in simian Vero E6 cells. This indicates that the degradation of the hantavirus Gn tail is not related to viral pathogenesis. We also report that the TULV Gn tails form aggresomes in transfected cells when treated with proteasome inhibitors.

METHODS

Cell cultures and reagents. HEK-293 (ATCC number CRL-1573) cells and Vero E6 cells were maintained in modified Eagle’s medium (Sigma) containing 10 % fetal bovine serum, 2 mM glutamine, 100 IU penicillin ml$^{-1}$ and 100 mg streptomycin ml$^{-1}$. The proteasomal inhibitors MG-132, ALLN and lactacystin, and protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma. Anti-green fluorescent protein (GFP) (Sigma), anti-beta-actin primary antibody (Sigma) and anti-haemagglutinin (HA) (Abcam) were used in immunoblotting and immunofluorescence.

Plasmid constructions and transfections. TULV, Puumala virus (PUUV) and PHV Gn tails were fused in-frame with enhanced GFP (eGFP) in the peGFP-C1 (Clontech) vector following PCR amplification with oligonucleotides containing EcoRI and SalI sites, and were verified by sequencing. The TULV Gn tail protein, lacking 21 or 34 residues from the C terminus, was fused to eGFP in the peGFP-C1 vector. Lipofectamine 2000 (Invitrogen) was used to transfect vectors into cells according to the manufacturer’s instructions.

Immunoblotting and fluorescence microscopy. Cells were collected and lysed in radioimmunoprecipitation (RIPA) buffer (150 mM sodium chloride, 50 mM Tris/HCl pH 7.5, 0.2 % SDS, 0.5 % deoxycholate, 1 % NP-40). Ten micromilligrams of total protein from each sample was run on 12 % SDS-PAGE and electroblotted onto a nitrocellulose membrane. Proteins were probed with an anti-GFP (Sigma) and anti-beta-actin primary antibody (Sigma) and a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (DAKO) and were detected by enhanced chemiluminescence.

For fluorescence detection of eGFP-tagged Gn tails, Vero E6 cells were transfected as described; fluorescence was analysed using an inverted fluorescence microscope (Olympus ZX17).
Confocal microscopy analysis. Cells were grown on coverslips in 24-well plates and transfected as described. Later, the cells were washed and fixed with 3.5% paraformaldehyde in PBS for 20 min at room temperature, permeabilized and blocked with 3% BSA/0.1% Triton X-100 in PBS. Mouse anti-HA antibody (Abcam), mouse antivimentin and mouse anti-β-tubulin was used as primary antibody, then after the staining with anti-mouse IgG red-fluorescent Alexa Fluor 555 conjugate (Invitrogen), cells were observed under a confocal microscope (Leica SP2).

Flow cytometry protocol. The cells were transfected as described and 24 h afterwards, the cells were treated overnight with 25 μM ALLN. Cells were collected, fixed with 3% paraformaldehyde (PFA) and washed with PBS. The percentage of GFP-positive cells after various treatments was calculated using flow cytometry (The Flow Cytometry Facility, Haartman Institute, University of Helsinki).

RESULTS AND DISCUSSION

To determine whether the Gn tail of TULV is proteasomally degraded, we fused the TULV Gn tail (residues 521–653) to the C terminus of eGFP in the peGFP-C1 vector (Fig. 1). Proteasome inhibition is known to stabilize proteins that are destined for degradation by the proteasome (Coux et al., 1996). Therefore, HEK-293 cells in 24-well plates were transfected with the eGFP-TULV-Gn tail

Fig. 2. (a) TULV Gn tail directs the eGFP-TULV-Gn tail to proteasomal degradation. HEK-293 were transfected with plasmid eGFP-TULV-Gn tail. Twenty-four hours later, the cells were treated overnight with (i) DMSO, (ii) ALLN, (iii) MG-132 or (iv) lactacystin. HEK-293 cells transfected with peGFP-C1 vector alone were used as a control and treated with (v) DMSO or (vi) ALLN. Live cells were observed under a fluorescence microscope. (b) TULV Gn tail colocalizes with ubiquitin. HEK-293 cells were cotransfected with eGFP-TULV-Gn tail and HA-tagged ubiquitin. Twenty-four hours later, the cells were treated with DMSO or ALLN overnight and then stained with antibody against HA, and analysed by confocal microscopy. (c) The C-terminal 21 residues of TULV Gn tail are required for degradation. eGFP-TULV-Gn tail del 34, eGFP-TULV-Gn tail del 21 and eGFP-TULV-Gn tail were expressed in HEK-293 cells. Twenty-four hours after transfection, the cells were treated with either ALLN (+) or DMSO (–) overnight. Cells were collected and lysed in RIPA buffer. Cell lysates were probed with an anti-GFP and anti-β-actin antibody.
construct. Twenty-four hours later, the cells were treated overnight with DMSO and proteasome inhibitors (25 μM ALLN, 10 μM MG-132 or 12.5 μM lactacystin dissolved in DMSO). We observed, by fluorescence microscopy, that the expression level of the eGFP-TULV-Gn tail was nearly undetectable when the cells were treated with DMSO, but that it increased after treatment with proteasomal inhibitors (Fig. 2a). These results suggested that proteasomal inhibitors stabilized the expression of the Gn tail fusion protein.

As proteins degraded by the proteasome are often associated with ubiquitination (Kopito, 2000), we tested whether the TULV Gn tail colocalizes with ubiquitin, as has been reported for the pathogenic NY-1 hantavirus (Geimonen et al., 2003). HEK-293 cells grown on coverslips in 24-well plates were cotransfected with eGFP-TULV-Gn tail and HA-tagged ubiquitin constructs (a gift...
from G. Kato, Johns Hopkins University). Twenty-four hours later, the cells were treated with DMSO or proteasome inhibitor ALLN overnight. Fixed cells were stained with mouse anti-HA antibody and then with anti-mouse IgG red-fluorescent Alexa Fluor 555 conjugate and observed under a confocal microscope. When treated with DMSO, the Gn tail of TULV was found in the perinuclear area, and the HA-tagged ubiquitin was found mostly in the nucleus and partially in the perinuclear region. When treated with a proteasomal inhibitor, the TULV Gn tail and ubiquitin colocalized in perinuclear structures resembling aggresomes (Fig. 2b), as discussed below. These results suggest that the Gn tail of TULV is directed to degradation through the ubiquitin-proteasome pathway, in a similar manner as described for pathogenic viruses (Geimonen et al., 2003; Sen et al., 2007).

Previous results have shown that the degradation signal (degron) for the NY-1 Gn tail resides within 5 and 30 residues from the C terminus (Sen et al., 2007). To check whether the TULV Gn tail behaves similarly, TULV Gn tail protein lacking 21 or 34 residues from the C terminus was fused to eGFP in the pEGFP-C1 vector (Fig. 1). HEK-293 cells were transfected with these constructs or the full-length eGFP-Gn tail. Twenty-four hours after transfection, the cells were treated with either 25 μM ALLN or DMSO overnight. Fig. 2(c) demonstrates that deletion of 21 or 34 residues from the C terminus completely stabilized the protein expression. These results indicate that the C-terminal 21 residues of TULV Gn tail are required for proteasomal degradation.

To confirm that Gn tails of other hantaviruses, apathogenic and pathogenic, are also degraded by proteasomes, we cloned the PHV Gn tail and PUUV Gn tail (Fig. 1). HEK-293 cells were transfected with eGFP-PHV Gn tail and eGFP-PUUV Gn tail, and the cells were treated separately with MG-132 or ALLN. Unexpectedly, the PHV hantavirus Gn tail was also degraded (Fig. 3), which contradicts previously reported results (Sen et al., 2007), while the PUUV Gn tail was degraded as expected (Fig. 3).

The results above showed that Gn tails of hantavirus were degraded by proteasomes in HEK-293 cells. We also tested the expression of Gn tails in simian Vero E6 cells and found that, in contrast with HEK-293 cells, the expression of the protein was easily detected by fluorescence microscopy and immunoblotting when cells were not treated with proteasome inhibitors. By using the protein synthesis and proteasome inhibitors, we showed that Gn tails of hantavirus were also degraded through the ubiquitin-proteasome pathway in Vero E6 cells. We transfected Vero E6 cells with eGFP-TULV-Gn tail, eGFP-PHV-Gn tail and eGFP-PUUV-Gn tail, and treated the cells with DMSO, CHX, MG-132 or MG-132 plus CHX. When we treated Vero E6 cells with the protein synthesis inhibitor CHX, Gn tail expression was abolished within 3 h (Fig. 4a), 5 h (Fig. 4b) and 4 h (Fig. 4c). When cells were treated with both CHX and MG-132, the level of the Gn tail protein remained stable. Compared with the DMSO-treated control, cells treated with the proteasome inhibitor MG-132 showed a clear increase in Gn tail protein level (Fig. 4). These results indicate that the detectable Gn tail protein level is maintained in Vero E6 cells by efficient synthesis. PHV Gn tail protein may be degraded somewhat more slowly and stay more stable than TULV Gn tail protein (TULV Gn tail protein was totally degraded in 3 h compared to PHV Gn tail protein which took 5 h when treated with CHX). It is clear that the three tested Gn tails are all degraded by proteasomes and the tail protein level is maintained by continuous synthesis and rapid degradation.

In order to compare the results from two different cell lines described above, we transfected Vero E6 and HEK-293 cells with eGFP-TULV-Gn tail and performed a FACS assay (Fig. 5). The cells were treated overnight with 25 μM ALLN, starting at 24 h post-transfection. Cells were
Fig. 6. (a) TULV Gn tail aggregates colocalize with vimentin and γ-tubulin. HEK-293 were transfected with eGFP-TULV-Gn tail plasmid and treated with 25 μM ALLN overnight; cells were stained with anti-vimentin or anti-γ-tubulin. Cells were observed by using Leica SP2 confocal microscopy. (b) Proteasomal inhibitors translocate Gn tail to aggresomes. HEK-293 cells were transfected with eGFP-Gn tail, and treated overnight with DMSO, MG-132 and/or NOC. The nuclei of attached cells were stained with Hoechst 33342 (Promega) (blue). Cells were observed under a fluorescence microscope. (c) The percentage of cells containing large aggresomes was determined from the ratio of large aggresome-containing cells to the number of transfected cells. At least 200 transfected cells were counted. Bars, SD.
collected, fixed with 3% PFA and washed with PBS. The percentage of GFP-positive cells after various treatments was calculated using flow cytometry (Fig. 5). Transfected HEK-293 cells treated with DMSO resulted in 6.69% of total cells being positive for GFP and those treated with ALLN resulted in 28% of total cells being GFP-positive. The corresponding results with Vero E6 cells showed that 11.5% (DMSO-treated) and 19.8% (ALLN-treated) were positive. When treated with DMSO, transfected Vero E6 showed a much higher expression level of Gn tail (11.5%) compared with HEK-293 cells (6.69%); this would be the reason why the Gn tail protein in transfected HEK-293 cells was much easier to detect than in transfected Vero E6 cells when not treated with ALLN.

As a result of overexpression, proteins may expose their hydrophobic surfaces, which normally will be buried in the protein’s tertiary or quaternary structure, due to inadequate folding or inadequate availability of chaperones. The exposure of hydrophobic sequences to an aqueous environment leads to the formation of aggregates degraded by the proteasome, and treatment of cells with proteasomal inhibitors has been shown to decrease the protein turnover when not treated with ALLN.

1998; Kopito, 2000). If the response of the unfolded protein to refold fails, the aggregates are transported to the microtubule-organizing centre (MTOC) (Diaz-Griffero et al., 2006; Kopito, 2000). This process clears the aggregates from the cytosol and concentrates them in a perinuclear structure called the aggresome. Aggresomes are often enveloped in vimentin cages and contain dynein, chaperones, ubiquitination enzymes and proteasomal components. When transfected HEK-293 cells were treated with a proteasome inhibitor, we found that the TULV Gn tail protein formed aggresome-like structures. These aggresome-like structures colocalized with ubiquitin (Fig. 2b).

To learn whether these structures are indeed aggresomes, we tested whether the TULV Gn tail protein colocalized with other proteins known to be associated with aggresomes. When transfected cells were treated with ALLN, TULV Gn tail colocalized with γ-tubulin, an MTOC marker, and vimentin, an aggresome marker (Fig. 6a).

Since aggresome formation is dependent on transport along microtubules, nocodazole (NOC; an anti-neoplastic agent that interferes with the polymerization of microtubules) can inhibit the formation of aggresomes leading to the dispersion of aggregates throughout the cytoplasm (Cardinale et al., 2001; Shi & Elliott, 2002). HEK-293 cells were seeded on coverslips and transfected with cGFP-Gn tail, and treated overnight with DMSO, 25 μM MG-132 and/or 1 μg NOC ml⁻¹. The nuclei of attached cells were stained with Hoechst 33342 (Promega) (blue). Cells were observed under a fluorescence microscope. As shown in Fig. 6(b), treatment of transfected HEK-293 cells with NOC together with MG-132 prevented the formation of MG-132-induced accumulation of TULV Gn tail protein into aggresomes and instead induced the dispersion of these structures diffusely to the cytoplasm. We counted the percentage of large aggresome-containing cells in transfected cells. In cells treated with both NOC and MG-132, the amount was lower than in cells treated only with MG-132 (Fig. 6c). These results show that, when overexpressed, TULV Gn tail translocates to the aggresomes formed.

To conclude, firstly, similar to pathogenic hantaviruses, the Gn tail of the apathogenic TULV was also degraded by the proteasome (Sen et al., 2007), and the hydrophobic C-terminal domain (or transmembrane helix) is the critical region leading to degradation. Notably, the reported lack of Gn tail degradation of the apathogenic PHV was based on simian COS7 cells (Sen et al., 2007) while we observed degradation of TULV and PHV Gn tails both in human HEK-293 and in simian Vero E6 cells. We conclude that the proteasomal degradation of hantavirus Gn tail is not related to viral pathogenesis. Secondly, the hantavirus Gn tail has a tendency to self-associate into large protein aggregates. It has been reported that when Sin Nombre virus (SNV) Gn full-length protein is expressed in the absence of Gc, it has the tendency to form SDS-stable oligomers (Spiropoulou et al., 2003). Such multimers could also be seen in immunoprecipitation experiments of the Hantaan virus-expressed Gn (Antic et al., 1992; Hooper et al., 2001; Pensiero & Hay, 1992) and the SNV Gn protein can be found in aggresomes when overexpressed (Spiropoulou et al., 2003). However, taken together, the results suggest that the degradation and aggregation of the envelope protein Gn may be a general property of most hantaviruses and is unrelated to pathogenicity.

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