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Maturation cleavage within the ectodomain of Lassa virus glycoprotein relies on stabilization by the cytoplasmic tail

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ABSTRACT

The Lassa virus glycoprotein consists of an ectodomain, a transmembrane anchor, and a cytoplasmic domain. It is synthesized as an inactive precursor and cleaved within the ectodomain to yield the mature form. Here, we show that this maturation cleavage can be abolished by mutation of single conserved amino acids within the cytoplasmic domain at the carboxy-terminus of the glycoprotein. Moreover, substitutions and deletions of multiple amino acids result in destabilization of the glycoprotein oligomers. These results indicate that conformation changes in the cytoplasmic domain travel across the membrane and subsequently abolish the maturation cleavage. Therefore, we postulate that the cytoplasmic domain is an important maturation factor stabilizing the overall conformation of the glycoprotein.

Structured summary:
MINT-7997004: *LASV GP* (uniprotkb:P08669) and *LASV GP* (uniprotkb:P08669) *physically interact* (MI:0915) by *cosedimentation through density gradient* (MI:0029)

1. Introduction

The *Arenaviridae* are divided into New World and Old World virus species. The latter one includes the prototypic family members lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV) [1]. The glycoprotein of LASV is synthesized as the precursor molecule preGP-C which is co-translationally processed by the cellular signal peptidase resulting in the 6 kDa stable signal peptide (SSP) and the immature 76 kDa GP-C. Uncommonly, after cleavage the SSP stays connected to the glycoprotein complex [2–4]. During the transport along the secretory pathway to the plasma membrane, GP-C is then further proteolytically activated by maturation cleavage into the distal glycoprotein subunit (GP-1) (44 kDa) and the transmembrane-spanning subunit (GP-2) (36 kDa) by the cellular endoprotease subtilase subtilisin kexin isozyme-1/site 1 protease (SKI-1/S1P) [5,6]. During this cleavage step, the SSP acts as an essential trans-acting maturation factor [3,7]. The glycoprotein forms spikes of trimers, in which the subunits are not covalently linked to each another [8]. In this report, we present data showing that single substitutions of conserved amino acids within the cytoplasmic domain of LASV glycoprotein (GP) result in the loss of maturation cleavage of GP-C. Moreover, glycoprotein oligomerization is disturbed by major mutations in the cytoplasmic domain. We postulate that the cytoplasmic domain acts as an essential stabilizing factor of the overall conformation of the glycoprotein which is of importance for the accessibility of the processing protease.

2. Materials and methods

2.1. Molecular cloning and protein expression

LASV GP (strain Josiah) cloned into pCAGGS vector was genetically modified by using recombinant PCR techniques and appropriate oligonucleotides as described before [7] with oligonucleotides available on request. The WE strain of LCMV was kindly provided by D. von Laer, Innsbruck, Austria.

Abbreviations: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; CD, cytoplasmic domain; CHO, Chinese hamster ovary; E-64, L-epoxysuccinyl-leucylamido (4-guanidino) butane; EDTA, ethylenediamine tetraacetate; GP, glycoprotein; GP-C, non-cleaved glycoprotein; GP-1, distal glycoprotein subunit; GP-2, membrane-anchored glycoprotein subunit; HA, hemagglutinin; LASV, Lassa virus; LCMV, lymphocytic choriomeningitis virus; MES, 2-(*N*-morpholino) ethanesulfonic acid; PBS, phosphate buffer saline; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gelelectrophoresis; SKI-1/S1P, subtilase kexin-isoenzyme/subtilase 1 protease; SSP, stable signal peptide; Tris, tris(hydroxymethyl)methylamine

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2.2. Cell culture, protein expression, acrylamide gel electrophoresis and immunoblotting

CHO-K1 (Chinese hamster ovary) cells were cultivated in DMEM:F12 medium (Invitrogen) under standard conditions [5]. Cells were transfected with plasmids containing cDNA of the viral glycoprotein constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. After 24 h of incubation, cells were lysed in sample buffer under reducing conditions and proteins were separated by sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS–PAGE) in 10% polyacrylamide gels. Immunoblotting was performed using a-GP-2-N rabbit antiserum, which binds the GP-2 subunit ectodomain [9]. Secondary labeling was performed with antibodies coupled with IRDye800 (Invitrogen) and proteins were visualized and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.3. Cell surface biotinylation

Glycoprotein-expressing cells were treated twice with 1 mg/ml sulfo-*N*-hydroxy-succinimidester-biotin (sulfo-NHS-biotin; Pierce) for 20 min at 4 °C and were then quenched by adding 0.1 M glycine for 5 min. The cells were lysed in phosphate buffer saline containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and Complete Protease Inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride, aprotinin, L-epoxysuccinyl-leucylamido (4-guanidino) butane, ethylenediamine tetraacetate, Leupeptin, [Sigma–Aldrich]). Then, biotinylated proteins were bound to streptavidin-coupled Sepharose beads (Pierce) and prepared for SDS–PAGE followed by immunoblotting [10].

2.4. Sucrose gradient ultracentrifugation

LASV GP expressing cells were lysed in MNT buffer (25 mM 2-(*N*-morpholino) ethanesulfonic acid pH 5.0; 100 mM NaCl; 30 mM tris(hydroxymethyl)methylamine (Tris)–HCl pH 7.4; protein inhibitors (cf. 2.3.) containing 1% Triton X-100. Pre-cleared cell lysates were ultracentrifuged in an SW60 rotor (Beckman) at 165 000×*g* for 18 h through a 5–35% (w/w) sucrose gradient containing 1% Triton X-100 in MNT. Gradients were fractionated from the top and samples of each fraction (200 µl) were subjected to SDS–PAGE and immunoblot analysis [8].

3. Results and discussion

3.1. The cytoplasmic domain of Lassa virus glycoprotein is essential for maturation cleavage

Amino acid sequence alignment of several arenavirus glycoproteins revealed 14 highly conserved (identical) and 2 semiconserved amino acids located within the cytoplasmic domain (Fig. 1A). We asked whether alterations of conserved amino acids influence glycoprotein maturation. For this purpose, we generated mutants of LASV GP with alanine substitutions of highly conserved amino acids, or deletions of either the C-terminal four amino acids (Δ487-491) or of the whole cytoplasmic domain (Δ451-491) (Fig. 1B). In addition, a chimeric glycoprotein was generated by replacement of the complete LASV cytoplasmic domain with that of the closely related LCMV GP (CD LCMV). We examined the maturation cleavage of GP-C after transient expression of plasmid-encoded wild type and mutated LASV GP in CHO cells by SDS–PAGE and immunoblot analysis (Fig. 1C). Interestingly, mutations of conserved amino acids led to abrogation of proteolytic processing, while the exchange of non-conserved amino acids, e.g. P483L, had no impact on GP-C cleavage. The low intensity seen for mutant

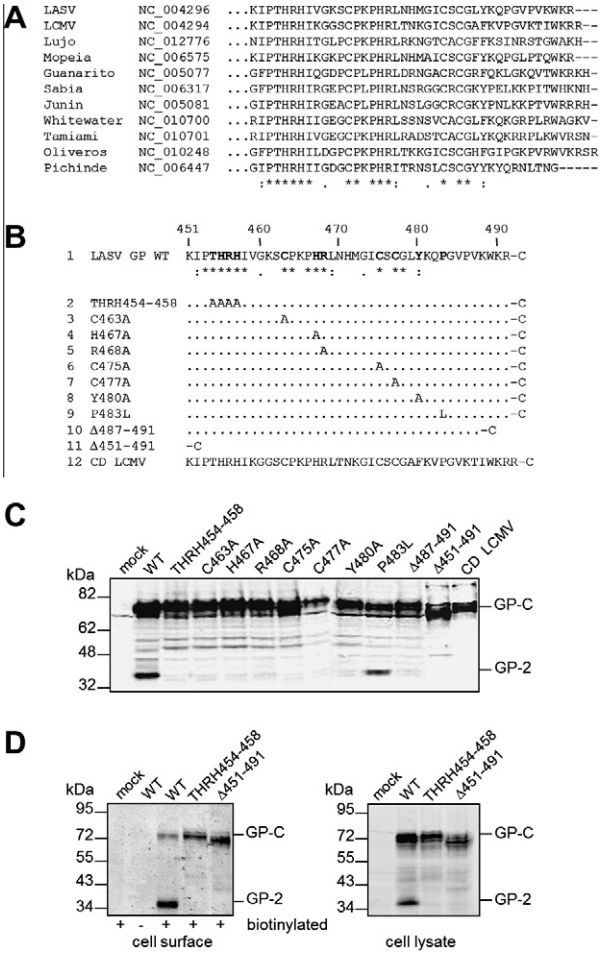

Fig. 1. Impact of the cytoplasmic domain on proteolytic activation of Lassa virus glycoprotein. (A) Amino acid sequences of LASV GP comprising the positions 451–491 and homologous sequences of other members of the arenavirus family listed with Genbank accession numbers were compared using Clustal W software. The symbols indicate identical (*) and semiconserved (.) amino acids as well as amino acids with similar properties (:). (B) Amino acid sequences of cytoplasmic domains (CD) of LASV GP wild type (line 1), substitution mutants (lines 2–9), deletion mutants (lines 10 and 11) and the replacement mutant CD LCMV (line 12) are shown. (C) Wild type and mutated LASV GP were recombinantly expressed in CHO cells, separated by SDS–PAGE and analyzed by immunoblotting. (D) Proteins on CHO cell surfaces were biotinylated, precipitated, and subjected to SDS–PAGE. LASV specific glycoprotein bands were detected by immunoblot analysis (left panel) and LASV GP expression of total cell lysates is shown in the right panel.

C477A was probably due to lower transfection efficiency. The deletion of the complete cytoplasmic domain (mutant Δ451-491) or a truncation of the basic C-terminal tetrapeptide (mutant Δ487-491) also resulted in abolished maturation cleavage. This is in agreement with observations obtained with LCMV by Kunz and coworkers but, interestingly, could not be detected in studies on the New world arenavirus Junin glycoprotein [4,11]. Surprisingly, our results also revealed that the chimeric LASV GP containing the cytoplasmic domain of LCMV (mutant CD LCMV) was not cleaved, although the cytoplasmic tail of LCMV GP does not differ in terms of the conserved amino acids. In contrast to the Lassa chimeric mutant CD LCMV the wild type LCMV is cleaved in CHO cells by SKI-I [12]. These data indicate that the conserved amino acids are not the only specification of the cytoplasmic domain which is necessary to support the maturation cleavage. We hypothesize that an accurate all-over conformation of the LASV cytoplasmic domain is required to obtain a correct conformation of the ectodomain which is an important prerequisite for cleavage accessibility

by the host protease. Apparently, the LCMV cytoplasmic domain fails to support this conformation.

3.2. Cytoplasmic domain mutants do not show impaired cell surface transport

It has been shown that both cleaved and non-cleaved wild type LASV glycoproteins are transported to the plasma membrane [13]. Moreover, in a previous report we demonstrated that cell surface transport of LASV GP is not dependent on its oligomeric state [8]. This observation is different from that of other viral glycoproteins, for instance, of influenza virus hemagglutinin and of glycoprotein G of vesicular stomatitis virus, which are only transported through the exocytic pathway in the correct oligomeric state [14,15]. To address the cell surface transport of the LASV GP in regard to alterations of the cytoplasmic domain we labeled selected mutants at the cell surface with non-membrane permeable biotin followed by precipitation with streptavidin-coupled Sepharose beads and immunoblotting. As shown in Fig. 1D, mutations within the cytoplasmic domain did not impact cell surface transport of GP-C. It is notable that cleaved GP is either transported more efficiently or is more stable at the cell surface. All LASV GP point mutants show the same apparent molecular mass, which indicates similar N-glycosylation supporting the result that the transport through the secretory pathway is not affected. The observed differences in the ratio of GP-C and GP-2 between biotinylated cell surface expressed GP and total cellular GP reflects the accumulation of the cleaved subunits at the cell surface while intracellularly the uncleaved precursor is predominantly detected. Taken together, this observation demonstrates that the abrogation of proteolytic activation described above is not due to impaired protein transport.

3.3. The cytoplasmic domain stabilizes non-cleaved GP-C for maturation cleavage

To investigate whether alterations of the cytoplasmic domain affect the quaternary structure of GP-C, we examined its oligomeric state. CHO cells expressing recombinant LASV GP were lysed and the proteins were separated in sucrose gradients by ultracentrifugation. The gradients were fractionated and each fraction was analyzed by immunoblotting. Wild type LASV GP-C was mainly detected in fractions 9–14, which correspond to the molecular masses of about 150–400 kDa as calculated from the marker proteins (Fig. 2A and B). The molecular mass of a monomeric GP-C molecule is 76 kDa. Consistent with our previous results [8], non-cleaved LASV GP-C comprises a wide range of oligomers from dimers up to hexamers. The substitution of four amino acids at positions 454–458 and the deletion of the whole cytoplasmic domain (Δ451-491) shifted the oligomerization pattern of GP-C to fractions 6–10 representing monomers and dimers. This altered oligomerization was not observed with LASV GP-C containing only single amino acid mutations, exemplified by mutant C475A (Fig. 2A). The much lower impact on the oligomerization by mutant C475A may be due to a minor conformational change of GP-C or to the loss of the interaction with a hypothetic intracellular binding partner that is required for proper processing of the GP-C ectodomain but not for trimer formation. However, these results show an important role of the cytoplasmic domain for the oligomerization of GP-C. We assume that the altered pattern of oligomerization results from conformational changes of the structure of GP-C.

One characteristic of LASV is that the signal peptide acts as an essential factor during maturation cleavage of the LASV glycoprotein [3], by a still unknown mechanism. To exclude any impact of the SSP on the results we observed in this work we investigated the contribution of the SSP on the oligomerization and stability of the glycoprotein complex. We performed sucrose gradient

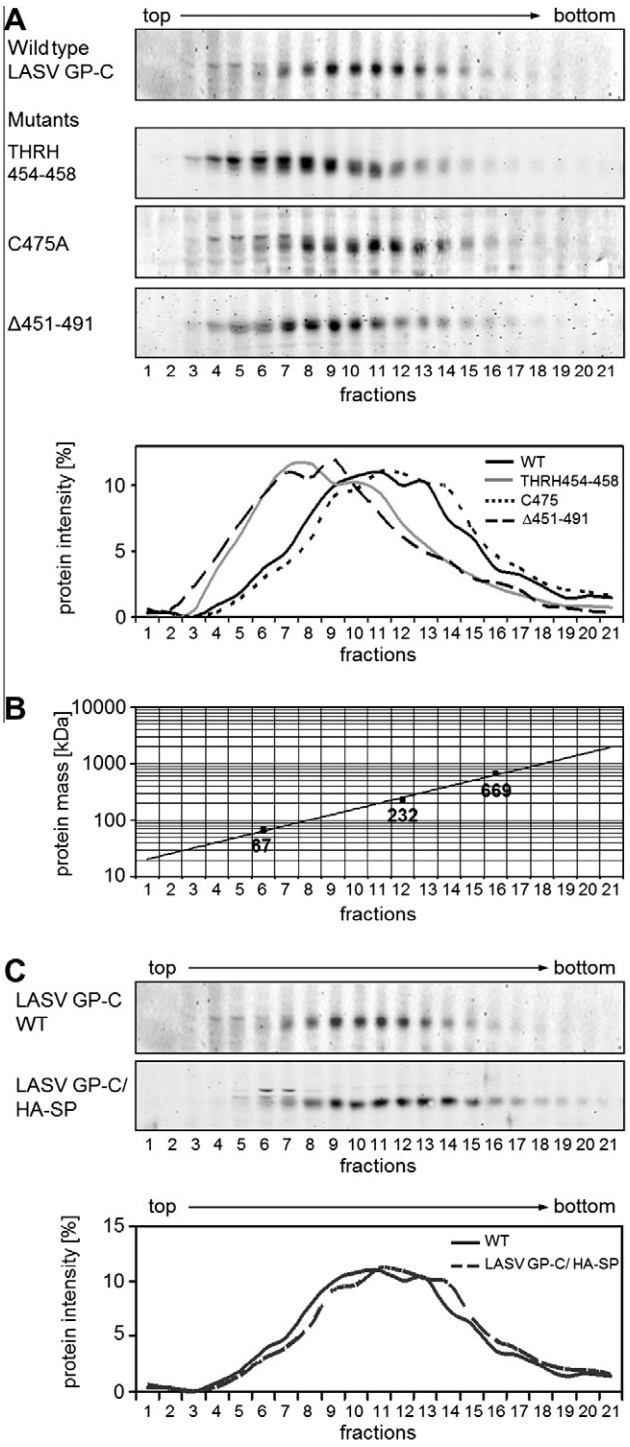

Fig. 2. Oligomerization of wild type and mutated Lassa viral glycoprotein. The state of oligomerization of wild type and LASV GP mutated in the cytoplasmic domain was investigated by sucrose gradient ultracentrifugation analysis and subsequent SDS–PAGE and immunoblotting. The relative protein intensities were graphed (A). The gradient marker proteins bovine serum albumin (67 kDa), catalase (232 kDa), and thyroglobulin (669 kDa) were examined in a parallel gradient and are indicated (B). (C) Comparison of the oligomeric states of wild type LASV GP and a chimera containing the signal peptide of influenza hemagglutinin (LASV GP-C/HA-SP).

centrifugation as described above of cells expressing a LASV GP mutant which contains the signal peptide of an influenza hemagglutinin protein (LASV GP-C/HA-SP). This mutant lacks its signal peptide since it has been previously shown that the signal peptide of HA does not stay connected to the LASV GP complex after signal

peptidase processing and does not support the cleavage of GP-C into GP-1 and GP-2 [3]. During sucrose gradient centrifugation the mutated proteins migrated in the same way as wild type LASV GP (Fig. 2C), both present primarily in fractions 9–14 showing that the SSP has no impact in the oligomerization of LASV GP-C. It is of note that this result does not allow any conclusions about the impact of the SSP on the stability of the mature, trimeric LASV GP-1/GP-2 complex after the maturation cleavage has occurred.

In conclusion, our data suggest that the cytoplasmic domain of the LASV glycoprotein substantially contributes to the stabilization of the quaternary glycoprotein structure, thus acting as an essential factor during LASV glycoprotein maturation. Many examples in the literature describe the cytoplasmic domain as important for the function of the ectodomains of glycoproteins in a variety of virus families [16–20]. Studies examining conformation dependent binding of monoclonal antibodies showed that alterations in the cytoplasmic domain led to structural changes in the ectodomain [21,22]. Moreover, our data highlight that the cytoplasmic domain is an important factor for a correct quaternary structure, which subsequently is required for proper proteolytic processing and function of the viral glycoprotein. Future investigations will show if the described changes in the ectodomain function of other viral glycoproteins also result from an impact of the cytoplasmic domain on the quaternary structure of the glycoprotein complexes.

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