Signal peptide of Lassa virus glycoprotein GP-C exhibits an unusual length

Robert Eichler, Oliver Lenz[1], Thomas Strecker, Wolfgang Garten*

Institut für Virologie der Philipps-Universität Marburg, Robert-Koch-Straße 17, D-35037 Marburg, Germany

Received 22 January 2003; accepted 6 February 2003

First published online 21 February 2003

Edited by Hans-Dieter Klenk

Abstract **Lassa virus glycoprotein is synthesized as precursor GP-C into the lumen of the endoplasmic reticulum and cleaved posttranslationally into the N-terminal subunit GP-1 and the C-terminal subunit GP-2 by subtilase SKI-1/S1P. The N-terminal portion of the primary translation product preGP-C contains a signal peptide of unknown length. In order to demonstrate the signal peptide cleavage site, purified viral GP-1 isolated from Lassa virus particles was N-terminally sequenced as TSLYKGV, identical to amino acids 59–65 of GP-C. Mutational analysis of the amino acid residues flanking the putative cleavage site led to non-cleavable preGP-C indicating that no other signal peptide cleavage site exists. Interestingly, GP-C mutants with a non-cleavable signal peptide were not further processed by SKI-1/S1P. This observation suggests that the signal peptide cleavage is necessary for GP-C maturation and hence for Lassa virus replication.**

Key words: Lassa virus; Signal peptide function; Glycoprotein maturation

1. Introduction

Signal peptides play a major role in sorting and membrane integration of secretory and membrane proteins [1]. Signal peptides are usually composed of three domains: an N-terminal highly variable domain with a usually net positive charge, a hydrophobic membrane-spanning domain comprising 6–15 amino acids, and normally a short C-terminal polar domain with small uncharged residues at the −1 and −3 position recognized by signal peptidase [2]. The total length of most signal sequences differs between 15 and 30 residues. Some signal sequences, however, show great variation in size and complexity. Over recent years, it has become clear that extended signal peptides may perform various additional functions besides the well known translocation of secretory and membrane-bound proteins across the membrane of the endoplasmic reticulum (ER). More and more examples of signal peptides, also of viral proteins, with additional biological significance have been investigated, for instance the signal peptide of the Foamy virus glycoprotein (GP) which is essential for particle egress of virions [3], the complete HIV gp120 signal peptide which retards the exocytosis of gp120 from the ER, and a proteolytic fragment of HIV gp120 signal peptide which binds to calmodulin. The functions of the latter two processes are not yet understood [4,5]. Another example is an immunodominant T-cell epitope within the signal peptide of the lymphocytic choriomeningitis virus (LCMV) GP that is conserved among all Arenaviridae [6,7].

Lassa virus, like LCMV, belongs to the large family of Arenaviridae comprising also other important human pathogens, like the viruses causing Argentine and Bolivian hemorrhagic fever. Lassa virus causes annually up to 100 000 cases of clinically apparent Lassa fever in West Africa, with 10–20% of the patients developing hemorrhagic manifestations and a total mortality of about 15% [8,9]. This disease is increasingly exported from endemic regions to other parts of the world [10].

The GPs of Lassa virus and LCMV have recently been shown to be posttranslationally processed by subtilase subtilisin kexin isoenzyme-1/site 1 protease (SKI-1/S1P) at a non-basic cleavage site which is unusual for fusogenic GPs of enveloped viruses [11–13]. However, the preceding cotranslational signal peptide cleavage of the Lassa virus GP has not been investigated so far. Neither the exact cleavage site(s), nor the real length of the signal peptide, nor the putative biological function(s) are known.

By determining the N-terminus of the GP subunit GP-1 and by mutating the potential cleavage site we could demonstrate that the signal peptidase cleaves only between threonine residues 58 and 59 of preGP-C. Surprisingly, preGP-C mutants containing non-cleavable signal peptide were not further proteolytically processed into GP-1 and GP-2, suggesting that the free signal peptide may have an additional function during virus replication beyond passing preGP-C through the ER membrane.

2. Materials and methods

2.1. Cell cultures and virus propagation

Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Experiments with Lassa virus

*Corresponding author. Fax: (49)-6421-286 8962.
E-mail address: garten@mailer.uni-marburg.de (W. Garten).

[1] Present address: European Molecular Biology Laboratory (EMBL) Grenoble Outstation, 6, rue Jules Horowitz, 38000 Grenoble, France.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GP, glycoprotein; KLH, keyhole limpet hemocyanin; PNGase F, peptide-*N*-glycosidase F; PVDF, polyvinylidene fluoride; SKI-1/S1P, subtilisin kexin isoenzyme-1/site 1 protease; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tricine, *N*-tris[hydroxymethyl]methylglycine

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00160-1

(strain Josiah) were performed under biosafety level 4 biocontainment conditions. Vero cells were infected with Lassa virus at a multiplicity of infection of 1. Cells were collected on day 4 post infection and infectious material was inactivated by boiling at 95°C for 10 min in presence of 1% sodium dodecyl sulfate (SDS) before viral protein was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. Purification of viral GP-1 and N-terminal amino acid analysis

The purification steps for GP-2 have been described in detail before [11]. Briefly, Lassa virus obtained from supernatants of virus-infected Vero cells and freed from cell debris by centrifugation (2000 rpm, 10 min) was pelleted through a 20% sucrose cushion by ultracentrifugation (20 000 rpm, 2 h, 5°C, SW28 rotor, Beckman) and further purified over a two-step Optiprep gradient (8%/30%) (Sigma). Viral protein of the gradient fractions was treated with peptide-*N*-glycosidase F (PNGase F), separated by gel electrophoresis (2.6), blotted onto a polyvinylidene difluoride (PVDF) membrane, and stained with Coomassie brilliant blue. The stained, deglycosylated GP-1 band was excised and the N-terminus determined by Edman degradation (WITA, Berlin-Teltow, Germany).

2.3. Mutation and vectorial expression of recombinant Lassa GP

The full-length GP and the signal peptide of Lassa virus (strain Josiah) were expressed using the β-actin promoter-driven pCAGGS vector [11,13]. Lassa Virus preGP-C mutants were generated by recombinant polymerase chain reaction using overlapping oligonucleotides, which will be made available on request [14]. Sequences were confirmed by DNA sequencing. Vero cells were transfected with wild type and mutated recombinant DNA using Lipofectamine 2000 (Gibco/Invitrogen).

2.4. Antibodies

Oligopeptides comprising the amino acids 2–18 and 477–491 of preGP-C were chemically synthesized and covalently linked to keyhole limpet hemocyanin (KLH, Pierce) as a carrier protein by the bifunctional agent *N*-[α-maleimidoacetoxy] succinimide ester. The oligopeptide–KLH was used for repeated immunization of rabbits as described before [11]. The obtained antisera (Rb-α-SP and Rb-α-GP2) were tested by peptide standard ELISA [15] and used for immunoprecipitation (Rb-α-SP) or immunoblot (Rb-α-GP2) analyses.

2.5. Acrylamide gel electrophoresis using Tris and tricine buffers, immunoblotting, and glycosidase treatment

Proteins were separated by two different methods: (i) SDS–PAGE as described by Laemmli [16] using 10% or 12% acrylamide gels, and (ii) SDS–PAGE as described by Schägger and von Jagow [17] using 16.5% polyacrylamide gel and *N*-tris[hydroxymethyl]methylglycine (tricine) buffer. Protein samples were dissolved in electrophoresis sample buffers containing either 100 mM Tris–HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue (four-fold) or 200 mM Tris–HCl (pH 6.8), 16% SDS, 8% 2-mercaptoethanol, 48% glycerol and 0.04% Serva blue G (four-fold). Immunoblotting was performed as described before [12]. Where indicated, samples were treated with PNGase F (New England Biolabs) at 37°C overnight.

2.6. Pulse-chase experiments, and immunoprecipitation

Plasmid-transfected Vero cells were starved 20 h post transfection for 1 h with DMEM lacking methionine and cysteine, before cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 30 min. The radioactive medium was replaced by DMEM for a 4-h chase. The labelled cells were lysed in radioimmunoprecipitation assay buffer and sonicated (40 W, Branson sonifier). Non-soluble material was removed and supernatants of the cell lysates incubated overnight with protein A-Sepharose-coupled antibodies. Precipitated immunocomplexes were subsequently analyzed by SDS–PAGE followed by autoradiography on BioMax films (Kodak).

3. Results and discussion

3.1. Identification of the N-terminus of Lassa virus GP-1

Several different signal peptide cleavage sites are possible for Lassa virus preGP-C. To locate the signal peptide cleavage site, we determined the N-terminus of the distal GP-1 subunit.

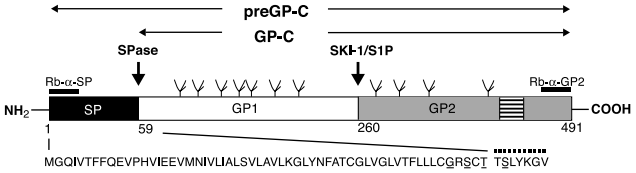

Fig. 1. Schematic overview of Lassa virus GP. The primary translation product preGP-C (aa 1–491), the signal peptide (SP) (aa 1–58), the precursor GP-C (aa 59–491) and the subunits GP-1 (aa 59–259) and GP-2 (260–491) containing the membrane anchor (stripes, aa 427–450) are shown. The antiserum binding sites, Rb-α-SP (aa 2–18) and Rb-α-GP2 (aa 477–491), the signal peptidase (SPase) cleavage site between threonine residues 58 and 59 (arrow), the SKI-1/S1P cleavage site C-terminal of leucine 259 (arrow), and putative *N*-glycosylation sites (tree-like symbols) are indicated. The total signal peptide sequence and C-terminally adjacent amino acid residues belonging to GP-1 are shown in one-letter code beneath. Single underlined amino acids are substituted in the signal peptide mutants. Amino acids overlaid with a striped line were determined by Edman degradation of GP-1.

GP-1 was purified from Lassa virus particles. The viral GPs were deglycosylated to obtain a sharp Coomassie blue-stainable band on the PVDF blot membrane and then subjected to SDS–PAGE and subsequently blotted. The band representing GP-1 was analyzed using Edman degradation. The first seven degradation cycles unambiguously revealed the amino acid sequence TSLYKGV (Fig. 1). This sequence is identical to the amino acids comprising threonine 59 to valine 65 of preGP-C, indicating that the signal peptide is cleaved after position 58.

3.2. Identification of the Lassa virus GP signal peptide

In order to prove or disprove the possibility that the signal peptide may be additionally cleaved elsewhere in the N-terminal direction, single amino acid residues flanking threonine 58 were substituted by large positively charged arginine residues. In general, signal peptide cleavage depends on small uncharged amino acids at positions −1 and −3 of the cleavage site and does not allow an arginine residue in these positions [2]. Thus we individually mutated the four small uncharged residues glycine 54, serine 56, threonine 58 and serine 60 of preGP-C, which are located around the cleavage site and could function as a recognition sequence for the signal peptidase, into arginine residues (Fig. 1). Recombinant wild type and mutated Lassa virus GP was then vectorially expressed in Vero cells and labelled radioactively in a pulse-chase experiment. The signal peptide, GP-C and preGP-C were detected by immunoprecipitation and autoradiography using the rabbit antisera Rb-α-SP and RB-α-GP2, respectively (Fig. 2). The released signal peptide of wild type and mutants G54R, S60R and as controls the recombinant signal peptide (aa 1–58) as well as material from Lassa-infected Vero cells were identified as bands with an apparent molecular mass of about 3.6 kDa on the 16.5% acrylamide gel (Fig. 2A). In contrast, released signal peptide of the mutants S56R and T58R was not detectable (Fig. 2A, lanes 3 and 4). Moreover, when radioactively labeled Lassa virus GP was precipitated with antibody Rb-α-GP2, deglycosylated and separated by 12% acrylamide electrophoresis, more slowly migrating bands appeared with mutants S56R and T58R in comparison to wild type, G54R and S60R. This suggests that the signal peptide was not or was only imperfectly split off from the primary gene product preGP-C of these mutants leading to a single band (mutant

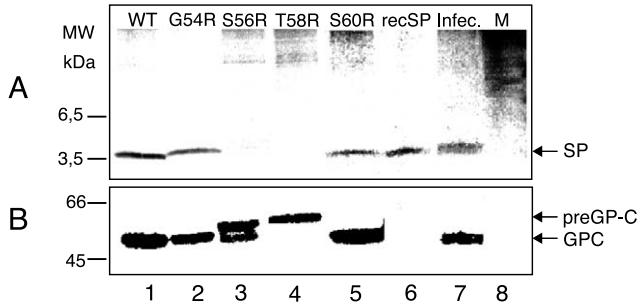

Fig. 2. Identification of the signal peptide cleavage site within Lassa virus GP. Vero cells were transfected with pCAGGS-LaGPC vector constructs containing wild type (WT), substitution mutants (G54R, S56R, T58R, and S60R), the recombinant signal peptide (pCAGGS-LaSP), or as a control with pCAGGS (M). The expressed proteins were either pulse-chase-labelled with [^{35}S]methionine/cysteine, immunoprecipitated (A) with the antiserum Rb-α-SP, and separated by SDS–tricine/PAGE and analyzed by autoradiography or (B) with Rb-α-GP2, treated with PNGase F and separated by SDS–PAGE on 12% acrylamide gels, and analyzed by autoradiography (B). For abbreviations see Fig. 1.

T58R) or a double band (mutant S56R) (Fig. 2B, lanes 3 and 4). The corresponding signal peptide band of the S56R mutant is apparently too weak to appear in Fig. 2A. These data demonstrate that Lassa virus GP possesses only one, 58 residues long signal peptide which is cleaved between threonine residues 58 and 59 with threonine 58 and serine 56 serving as the recognition motif for the eukaryotic signal peptidase. The sequenced N-terminus of LCMV GP-1 indicated that signal peptide cleavage occurs at the homologous position of preGP-C, although the amino acids at the cleavage site are not identical [6].

Using different durations of chase in pulse-chase experiments we also found that complete cleavage of the signal peptide can be detected already after a 10 min pulse with the wild type and mutants G54R and S60R, while for mutants T58R and S56R even after 15 h of chase no or only very little cleavage was detectable, indicating that cleavage of Lassa virus preGP-C occurs only cotranslationally. Interestingly, the released signal peptide was still detected after 15 h of chase. In analogy, the LCMV GP-C signal peptide is also very stable and can still be detected after several hours (M. Froeschke, M. Groettrup and B. Dobberstein, personal communication). In addition, an in vitro-transcribed and translated signal peptide migrates to the same position on acrylamide gels indicating that the released signal peptide is not processed further (data not shown). Taken together we show that the released full-length signal peptide is cotranslationally released and not degraded immediately after cleavage from preGP-C like usual signal peptides. We therefore suggest that the Lassa virus GP signal peptide may have an additional function.

3.3. *Signal peptide cleavage is essential for maturation cleavage of GP-C*

The highly extended length of the signal peptide with 58 residues leads to the question of the use of such an unusually long signal peptide. In an attempt to reveal possible further functions of the signal peptide apart from ER translocation, we addressed the question whether the release of the signal peptide is necessary for the maturation cleavage of GP-C by SKI-1/S1P. For this purpose, we analyzed wild type GP-C and the signal peptide cleavage site mutants with respect to

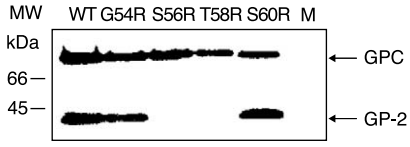

Fig. 3. Proteolytic processing of Lassa virus GP by SKI-1/S1P. Vero cells were transfected with wild type (WT), mutants of the recombinant Lassa virus GP and mock-transfected (M). Virus GP was separated on 10% acrylamide gels by SDS–PAGE and immunoblotted. Non-cleaved GP-C and its cleaved form GP-2 are detected by immunostaining using the antiserum Rb-α-GP2.

the maturation cleavage of GP-C into GP-1 and GP-2 by SKI-1/S1P using the antiserum Rb-α-GP2 which recognizes the non-cleaved GP and the membrane-anchored GP-2, but not GP-1. GP-2 is not detectable in the signal peptide cleavage-defective mutants T58R and S56R, whereas the other substitution mutants (mutants G54R and S60R) do not affect the maturation cleavage of GP-C into GP-1 and GP-2 (Fig. 3).

Since SKI-1/S1P resides in the ER, it is surprising to see that preGP-C, which should also be present in the ER, is not cleaved by SKI-1/S1P, whereas GP-C is cleaved by SKI-1/S1P before it reaches the medial Golgi [12]. Thus, cleavage of the signal peptide could be a prerequisite for the transport of the GP precursor to a late ER or early Golgi compartment where the maturation cleavage could occur. This explanation appears unlikely since preliminary data indicate that mutant preGP-C is at least transported to the Golgi complex (data not shown). Another explanation could be the masking of the maturation cleavage site C-terminally of amino acid 259 by the covalently bound signal peptide. It is also conceivable that preGP-C is not folded correctly and thus is not exposing the maturation cleavage site to SKI-1/S1P.

However, our data clearly show that the signal peptide cleavage is a primary essential step before the secondary step, the maturation cleavage of GP-C, can occur. Whether the cleavage process of the signal peptide itself or the released signal peptide, for instance as an intramolecular chaperone, plays the crucial role for the secondary step, the cleavage of GP-C by SKI-1/S1P, is currently under investigation. The latter possibility is favored by the unusual length of the signal peptide which comprises a wide range of predominantly hydrophobic amino acid residues indicating that this peptide might be responsible for other functions.

Since activation cleavage of GP-C by SKI-1/S1P is a prerequisite for the generation of infectious virions, the specific inhibition of the Lassa virus signal peptide cleavage which is essential for the maturation cleavage may also be discussed as a potential therapeutic target. This possible therapeutic concept and the unusual length of the signal peptide make the study of its function and structure, as well as the elucidation of its viral and cellular interaction partners, highly interesting.

Acknowledgements: We thank H.D. Klenk, M. Eickmann and J. ter Meulen for stimulating discussions. Peptides were synthesized and kindly provided by M. Krause, Institut für Molekularbiologie und Tumorforschung, Universität Marburg. This work was supported by the Deutsche Forschungsgemeinschaft, Sachbeihilfe Ga 282/4-1 and SFB 286 TP1 and the Graduiertenkolleg 'Protein Function at the Atomic Level'. R.E. was supported by the FAZIT-Stiftung, Frankfurt, and performed this work in partial fulfilment of the requirements for a Ph.D. degree from the Philipps-Universität Marburg.

References

[1] Martoglio, B. and Dobberstein, B. (1998) Trends Cell Biol. 8, 410–415.
[2] von Heijne, G. (1985) J. Mol. Biol. 184, 99–105.
[3] Lindemann, D. et al. (2001) J. Virol. 75, 5762–5771.
[4] Li, Y., Bergeron, J.J., Luo, L., Ou, W.J., Thomas, D.Y. and Kang, C.Y. (1996) Proc. Natl. Acad. Sci. USA 93, 9606–9611.
[5] Martoglio, B., Graf, R. and Dobberstein, B. (1997) EMBO J. 16, 6636–6645.
[6] Burns, J.W. and Buchmeier, M.J. (1993) in: The Arenaviridae (Salvato, M.S., Ed.), pp. 17–33, Plenum Press, New York.
[7] Hombach, J., Pircher, H., Tonegawa, S. and Zinkernagel, R.M. (1995) J. Exp. Med. 182, 1615–1619.
[8] McCormick, J.B., Webb, P.A., Krebs, J.W., Johnson, K.M. and Smith, E.S. (1987) J. Infect. Dis. 155, 437–444.
[9] McCormick, J.B., King, I.J., Webb, P.A., Johnson, K.M., O'Sullivan, R., Smith, E.S., Trippel, S. and Tong, T.C. (1987) J. Infect. Dis. 155, 445–455.
[10] ter Meulen, J., Lenz, O., Koivogui, L., Magassouba, N., Kaushik, S.K., Lewis, R. and Aldis, W. (2001) Trop. Med. Int. Health 6, 83–84.
[11] Lenz, O., ter Meulen, J., Feldmann, H., Klenk, H.D. and Garten, W. (2000) J. Virol. 74, 11418–11421.
[12] Lenz, O., ter Meulen, J., Klenk, H.D., Seidah, N.G. and Garten, W. (2001) Proc. Natl. Acad. Sci. USA 98, 12701–12705.
[13] Beyer, W.R., Pöpplau, D., Garten, W., von Laer, D. and Lenz, O. (2003) J. Virol. 7, 2866–2872.
[14] Higuchi, R., Krummel, B. and Saiki, R.K. (1988) Nucleic Acids Res. 16, 7351–7367.
[15] Munk, K., Pritzer, E., Kretzschmar, E., Gutte, B., Garten, W. and Klenk, H.D. (1992) Glycobiology 2, 233–240.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.