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Long-lived Signal Peptide of Lymphocytic Choriomeningitis Virus Glycoprotein pGP-C*[S]

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Marc Froeschke‡, Michael Basler§, Marcus Groettrup§, and Bernhard Dobberstein‡¶

From the ‡Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg and the §University of Constance, Department of Immunology, Universitätsstrasse 10, D-78457 Konstanz, Germany

Signal peptides (SPs) direct nascent secretory and membrane proteins to the membrane of the endoplasmic reticulum. They are usually cleaved from the nascent polypeptide by signal peptidase and then further proteolytically processed. The SP of the pre-glycoprotein (pGP-C) of the lymphocytic choriomeningitis virus SP^{GP-C} (signal peptide of pGP-C) shows different properties: 1) The SP^{GP-C} is unusually long (58 amino acid residues) and contains two hydrophobic segments interrupted by a lysine residue. 2) The SP^{GP-C} is cleaved only from a subset of pGP-C proteins. A substantial portion of pGP-C accumulates that still contains the SP^{GP-C}. 3) The cleaved SP^{GP-C} is rather long-lived ($t_{1/2}$ of more than 6 h). 4) The cleaved SP^{GP-C} resides in the membrane and is resistant to digestion with proteinase K even in the presence of detergents, suggesting a very compact structure. 5) SP^{GP-C} accumulates in virus particles. These unusual features of the cleaved SP^{GP-C} suggest that SP^{GP-C} not only targets the nascent pGP-C to the endoplasmic reticulum membrane but also has additional functions in lymphocytic choriomeningitis virus life cycle.

Most secretory and membrane proteins are synthesized as preproteins with an N-terminal signal peptide (SP)[1] (1, 2). Signal peptides are usually 15–25 amino acid residues in length and are typically comprised of three distinct regions: a central hydrophobic core of 7–10 residues (h-region), a polar N-terminal region that can be very variable in length and is usually positively charged, and a C-terminal region that contains the cleavage site for the signal peptidase (3). In eukaryotes, SPs target nascent secretory and membrane proteins to the endoplasmic reticulum (ER) and mediate insertion of the nascent polypeptides into the translocon (4). Membrane insertion is thought to occur in a loop-like fashion such that the N terminus of the SP is exposed on the cytoplasmic side of the membrane. Signal peptidase then cleaves the SP on the lumenal side of the membrane (5, 6). Cleavage usually occurs cotranslationally; however in some cases, SP cleavage is delayed or does not occur at all. Delayed cleavage is observed for the US11 SP (7) and the SP of the human immunodeficiency virus-1 (HIV-1) glycoprotein 160 (gp160) (8). The SP of the human cytomegalovirus (HCMV) US2 gene product is not even cleaved at all (9). Mutational analyses of several preproteins revealed that the efficiency and fidelity of SP cleavage can be influenced by mutations within the signal sequence itself but also by mutations in the mature protein (10–12).

After cleavage from the preprotein, SPs are thought to be either directly degraded or processed by signal peptide peptidase (SPP) into distinct fragments that are released from the membrane (13, 14). SPP has recently been shown to be a presenilin-type intramembrane-cleaving protease (15). Several determinants for processing by SPP have been identified: the SP has to be cleaved from the preprotein and the hydrophobic core region has to contain helix-breaking residues. Moreover, as yet ill-defined features of the SP flanking regions can affect SP processing (16).

SP fragments resulting from processing by SPP can be functionally active. In the case of the hormone preprolactin and the human immunodeficiency virus-1 (HIV-1) gp160, the N-terminal portion of the respective SP is released into the cytosol and binds to calmodulin in a Ca^{2+}-dependent manner (17). This implies that these SP-derived fragments may influence signal transduction pathways in the cell (17).

SPs can also play a role in immunorecognition. SPs of the polymorphic major histocompatibility complex (MHC) class I molecules contain a highly conserved sequence that is capable of binding to nonpolymorphic MHC class I molecules (HLA-E in human) (18). The peptide-HLA-E complex interacts at the cell surface with an inhibitory receptor on natural killer cells and thereby monitors the level of MHC class I molecule expression (19). Presentation of the SP-derived epitope is dependent on the transporter associated with antigen processing that transports peptides generated by the proteasome into the ER lumen (20).

The glycoproteins (GP-1 and GP-2) of the lymphocytic choriomeningitis virus (LCMV) are synthesized as a type I precursor glycoprotein C (pGP-C) that is processed during intracellular transport into GP-1 and GP-2 (21). The predicted SP of LCMV pGP-C (SP^{GP-C}) is rather unusual: it is longer than average SPs, comprising 58 amino acid residues, and has two hydrophobic regions separated by a lysine residue. An epitope derived from the core region, amino acid residues 33–41/43 of the SP^{GP-C} (gp33), is presented by MHC class I molecules to cyto-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplementary figures 1S–3S showing Western blot analysis of supernatant and pellet fractions after cell fractionation, Western blot analysis of L929 cells and purified virus particles, and sequence alignment of the predicted signal sequences of some arenavirus glycoproteins.

¶ To whom correspondence should be addressed: Tel.: 49-6221-546825; Fax: 49-6221-545892; E-mail: dobberstein@zmbh.uni-heidelberg.de.

[1] The abbreviations used are: SP, signal peptide; SPP, signal peptide peptidase; pGP-C, pre-glycoprotein C; pGP-C-HA, C-terminal HA-tagged pGP-C; $pGP\text{-}C_{142}$, truncated (amino acid residues 1–142) pGP-C; SP^{GP-C}, signal peptide of pGP-C; ER, endoplasmic reticulum; HA, hemagglutinin; HCMV, human cytomegalovirus; LCMV (WE), lymphocytic choriomeningitis virus strain WE; gp, glycoprotein; HIV, human immunodeficiency virus; HIV-1 gp160, glycoprotein 160 of HIV-1; PNGase F, protein *N*-glycanase F; MHC, major histocompatibility complex; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

toxic T lymphocytes (21–24). Presentation requires a functional transporter associated with antigen processing (25) and proteasome activity (26). We have investigated the cleavage and fate of the SP^{GP-C} in transfected and LCMV-infected cells.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmid pSV51l/pGP-C was derived from the widely used original cDNA of LCMV (WE) S RNA (27) and the pSV51l expression vector (28). A DNA fragment including the entire coding region for LCMV (WE) pGP-C (GenBank™ accession number M22138: base pairs 17–1,579) was cloned into pSV51l using an additionally introduced *Bam*HI site (pSV51l/pGP-C).

A DNA fragment encoding pGP-C-HA was generated by polymerase chain reaction using the forward primer 5′-GGA TCT CTA GAG TCG ACC CC-3′ and the backward primer 5′-CTG GAT CCT CAA GCG TAA TCT GGA ACA TCG TAT GGG TAG CGT CTT TTC CAG ATA G-3′. The PCR product was inserted into the *Bam*HI site of pSV51l (pSV51l/pGP-C-HA).

Cells and Virus—HeLa and L929 cells were obtained from American Type Culture Collection (Manassas, VA). MC57 cells have been described previously (29). HeLa, MC57, and L929 cells were grown under recommended conditions. The LCMV (WE) strain was originally obtained from F. Lehmann-Grube (30).

Peptides and Antibodies—Synthetic peptides deduced from the SP^{GP-C} and including amino acid residues 7–18 (MFEALPHIIDEV, SP7), 30–41 (TSIKAVYNFATC, SP30), and 48–57 (SFLFLAGRSC, SP48) were coupled to keyhole limpet hemocyanin and injected into rabbits to raise anti-SP7, -SP30, and -SP48 antibodies. Antisera were immunopurified. KL25 is a mouse monoclonal antibody reactive with the LCMV glycoprotein GP-1 (31).

Transfection, Infection, and LCMV Particle Preparation—Expression vectors were transfected into HeLa cells by the calcium phosphate precipitation method for 20–24 h (32). The cells were grown for an additional 48 h. MC57 cells were infected for 24 h with LCMV (WE) (multiplicity of infection, 0.05). LCMV particles were prepared as described (33) without using a continuous sucrose gradient.

Metabolic Labeling and Immunoprecipitation—After transfection, about 1×10^6 cells were starved in Met/Cys-free Dulbecco's modified Eagle's medium plus 10% fetal calf serum for 150 min at 37 °C and labeled with 0.15 mCi/ml [^{35}S]Met/Cys for 30 min. Labeling medium was removed, and cells were washed with phosphate-buffered saline and either directly analyzed or chased in complete Dulbecco's modified Eagle's medium for the indicated time periods.

Cells were harvested and lysed for 15 min on ice in 100 mM NaCl, 20 mM HEPES/KOH (pH 7.3), 5 mM $MgCl_2$, 1% (w/v) Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Equal aliquots were used for immunoprecipitation (34).

After infection 2×10^6 confluent cells were starved in Met/Cys-free RPMI 1640 plus 10% dialyzed fetal calf serum for 45 min at 37 °C and labeled with 0.1 mCi/ml [^{35}S]Met/Cys for 60 min. Labeling medium was removed, and cells were washed with phosphate-buffered saline and chased in complete medium for the indicated time periods. Cells were harvested and lysed for 45 min on ice in 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.85 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2.8 μM pepstatin, 0.75 μM aprotinin, and 2% (v/v) Nonidet P-40. Equal aliquots were used for immunoprecipitation. The beads were boiled in the appropriate sample buffer (35, 36), and proteins were then analyzed by SDS-PAGE followed by phosphorimaging using a BAS 1500 (Fuji, Tokyo, Japan). Protein amounts have been quantified using MacBas2.0 program.

Cell Fractionation and Treatments of Isolated Membranes—Transfected HeLa cells were harvested, resuspended in 20 volumes of hypotonic buffer (5 mM HEPES/KOH (pH 7.4), 0.5 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin), incubated on ice for 10 min, and broken up by using a Dounce homogenizer. The homogenate was adjusted to 0.25 M sucrose and centrifuged at 4 °C, 13,800 × *g* for 5 min. The supernatant was loaded on a high salt sucrose cushion (500 mM sucrose, 500 mM KOAc, 50 mM HEPES/KOH (pH 7.6), 2 mM MgOAc, 1 mM dithiothreitol, and 10 mg/ml phenylmethylsulfonyl fluoride) and centrifuged at 4 °C, 100,000 × *g* for 30 min. The resulting pellet (membranes) was resuspended in hypotonic buffer, adjusted to 100 mM NaCl and 0.25 M sucrose, and aliquots were treated on ice with different combinations of 0.5 mg/ml proteinase K, 0.5 mg/ml trypsin, 1% (w/v) Triton X-100, or increasing concentrations of SDS (0.2, 0.5, and 1.0%) as indicated in the legend for Fig. 4.

For carbonate extraction, one aliquot of membranes was treated with 1/10 volume of 1 M Na_2CO_3 (pH 11) for 15 min on ice and loaded on a sucrose cushion (250 mM sucrose, 100 mM Na_2CO_3, pH 11). After centrifugation (4 °C, 130,000 × *g*, 10 min), the supernatant was taken off, and the pellet was resuspended in hypotonic buffer adjusted to 100 mM NaCl and 0.25 M sucrose.

PNGase F Treatment of Immunoprecipitated Proteins—Immunoprecipitated proteins bound to resin were incubated with 100 units of PNGase F (New England Biolabs, Schwalbach, Germany) in a final volume of 30 μl as specified by the supplier and incubated for 12 h at 37 °C.

Western Blot Analysis—Proteins of total cell lysates, fractions of membranes, cytosol, or LCMV particles were separated by SDS-PAGE, transferred to nitrocellulose, and identified using anti-SP7, anti-Sec61β, or anti-α-tubulin antibodies by standard Western blot analysis technique (32).

RESULTS

Characterization of Antibodies Directed against the pGP-C Signal Peptide (SP^{GP-C})—To characterize the SP of the LCMV glycoprotein pGP-C (SP^{GP-C}) (Fig. 1*A*), antibodies against three different segments of the SP^{GP-C} were raised in rabbits. The peptides used for immunization comprised amino acid residues 7–18 (SP7), 30–41 (SP30), and 48–57 (SP48) (Fig. 1*B*). Antibodies were affinity-purified and used to immunoprecipitate a fragment of pGP-C, comprising amino acid residues 1–142 ($pGP\text{-}C_{142}$) (Fig. 1*A*), which was synthesized *in vitro*. An aliquot of the translation reaction (Fig. 1*C*, *lane 1*) and the immunoprecipitates (*lanes 2–13*) were separated by SDS-PAGE, and $pGP\text{-}C_{142}$ was visualized by phosphorimaging. Only the anti-SP7 antibody was able to immunoprecipitate $pGP\text{-}C_{142}$ (Fig. 1*C*, *lane 2*). The specificity of the reaction was demonstrated by the addition of the SP7 or an unrelated (control) peptide. No immunoprecipitation is seen when the specific peptide SP7 is included in the reaction (*lane 3*), whereas a control peptide had no effect (*lane 4*). The preimmune serum did not immunoprecipitate $pGP\text{-}C_{142}$ (*lane 5*). Antibodies raised against the other two SP peptides, SP30 and SP48, did not immunoprecipitate $pGP\text{-}C_{142}$ (*lanes 6–13*). Anti-SP7 antibody was used for the further studies.

Identification of (p)GP-C-HA and SP Cleavage from pGP-C—SPs are usually cleaved very rapidly after their membrane insertion from the nascent or just completed secretory or membrane protein. To follow cleavage of the SP^{GP-C} from pGP-C, we initially used a cell-free system in which $pGP\text{-}C_{142}$ was synthesized in the presence of rough microsomal membranes (13). Cleavage of the SP^{GP-C} from $pGP\text{-}C_{142}$ was very inefficient as compared with SP cleavage from the secretory protein preprolactin (data not shown). We therefore reasoned that SP^{GP-C} cleavage from pGP-C might be inherently inefficient. To test this hypothesis, we transfected HeLa cells with a plasmid expressing HA epitope-tagged LCMV pGP-C (pGP-C-HA) (Fig. 1*A*). Cells were labeled with [^{35}S]Met/Cys for 30 min. The extract was treated with PNGase F to remove *N*-linked carbohydrates from pGP-C-HA and thereby obtain a more distinct banding pattern. Unglycosylated (p)GP-C-HA ((p)GP-C-HA*) was immunoprecipitated with anti-HA or anti-SP7 antibodies (Fig. 2*A*). Both antibodies immunoprecipitated a major band of about 55 kDa, the expected molecular mass of unglycosylated (p)GP-C-HA, (p)GP-C-HA* (*lanes 3* and *4*). In the anti-HA immunoprecipitates, we cannot detect a smaller form representing mature GP-C-HA*, suggesting either that pGP-C-HA* and GP-C-HA* are not resolved by the gel system used or that GP-C-HA* is degraded. In addition, the anti-SP7 antibody immunoprecipitates a peptide of about 6 kDa. The 6-kDa peptide comigrates with *in vitro* synthesized SP^{GP-C} comprising the N-terminal 58 amino acid residues of pGP-C (SP58) (Fig. 2*B*, *lane 2*). This suggests that the complete SP^{GP-C} accumulates.

To investigate whether the SP^{GP-C} is stable enough to be detected under steady state conditions, we used Western blot-

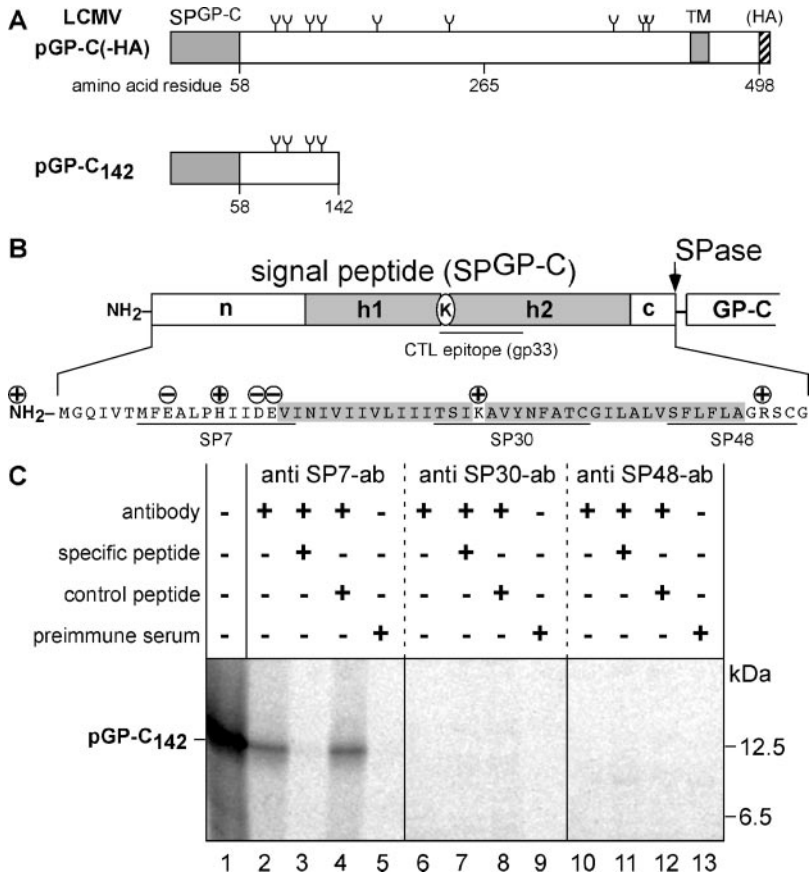

FIG. 1. *A*, outline of LCMV pGP-C tagged at its C terminus with a HA tag (pGP-C-HA). The signal peptide (SP^{GP-C}), the transmembrane region (*TM*), and the HA tag (HA) are indicated. The *forked symbols* indicate potential sites for *N*-glycosylation. $pGP\text{-}C_{142}$ is a fragment of pGP-C synthesized in an *in vitro* system from a truncated pGP-C mRNA. *B*, outline of SP^{GP-C}. The N-terminal (*n*), hydrophobic (h1, h2), the C-terminal (*c*) regions, and the site for signal peptidase (*SPase*) cleavage are indicated. The position of the lysine residue between the h1 and h2 region (*K*) and positively (+) and negatively (−) charged amino acid residues in the SP^{GP-C} are indicated. The immunodominant epitope of cytotoxic T lymphocytes (CTL), gp33 and peptide sequences (SP7, SP30, and SP48) that were used to raise antibodies are *underlined*. *C*, characterization of anti-SP7, -SP30, and -SP48 antibodies by immunoprecipitation of *in vitro* synthesized $pGP\text{-}C_{142}$. Proteins were separated on a 15% SDS gel and $pGP\text{-}C_{142}$ visualized by phosphorimaging. The respective specific peptides or unrelated peptides were added as indicated.

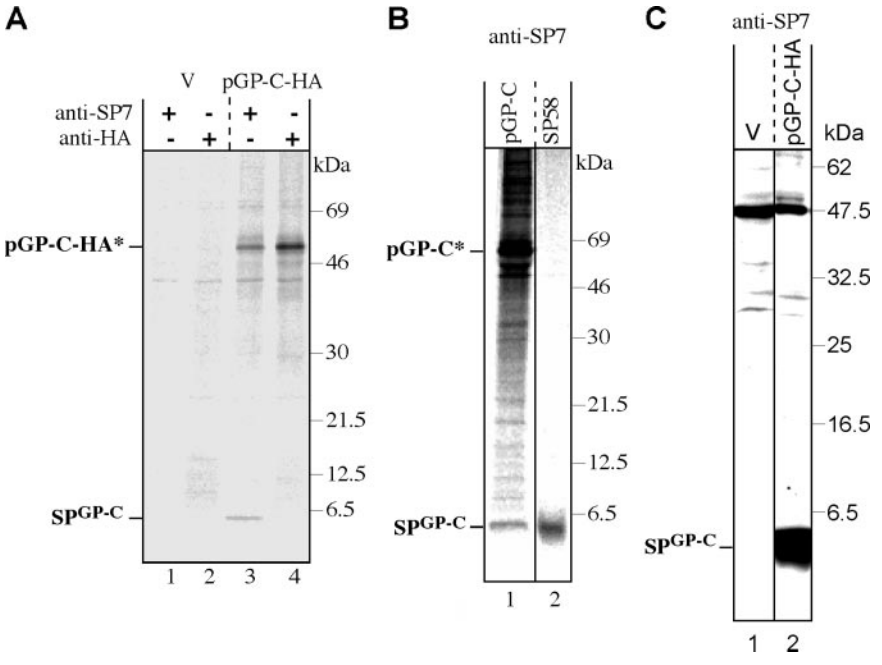

FIG. 2. **Identification of pGP-C(-HA) and SP^{GP-C} in transfected HeLa cells.** In *A*, HeLa cells were transfected with plasmid pSV51l (*V*) or pSV51l/pGP-C-HA and labeled for 30 min. with [^{35}S]Met/Cys. Antigens were immunoprecipitated with anti-SP7 or anti-HA antibodies and PNGase F-treated. Proteins were separated by SDS-PAGE, and unglycosylated pGP-C-HA* and SP^{GP-C} were visualized by phosphorimaging. *B*, size estimation of SP^{GP-C}. Transfected cells expressing SP^{GP-C} were analyzed as in *A* (*lane 1*). To obtain a molecular size standard of the SP^{GP-C}, we translated a truncated mRNA coding for the N-terminal 58 amino acids of pGP-C (SP58) (*lane 2*). SP^{GP-C} and *in vitro* synthesized SP58 comigrate (*cf. lanes 1* and *2*). *C*, identification of SP^{GP-C} by Western blotting. Transfected cells expressing pGP-C-HA as described in *A* were prepared for Western blotting using a Tricine type gel and probed with anti-SP7 antibody. SP^{GP-C} is seen in cells expressing pGP-C-HA (*lane 2*) but not in cells transfected with control vector (*V*) (*lane 1*).

ting. A cell extract from transfected HeLa cells was separated on an SDS-PAGE Tricine gel that separates peptides with high resolution, and a Western blot was probed with the anti-SP7 antibody. As can be seen in Fig. 2*C*, the SP^{GP-C} can be detected in cells expressing pGP-C-HA (*lane 2*) but not in cells transfected with the empty vector (*lane 1*).

Stability of the Cleaved SP^{GP-C}—To follow cleavage of the SP^{GP-C} from pGP-C-HA, we pulse-labeled transfected HeLa cells for 30 min and chased them with medium containing unlabelled amino acids for 3 and 6 h (Fig. 3*A*). Aliquots of the samples were treated with PNGase F as indicated in the figure. The anti-SP7 antibody was used to immunoprecipitate pGP-C-HA and the cleaved SP^{GP-C}. SP7 or control peptides were added to the samples as indicated. After the labeling and PNGase F treatment, the 55-kDa pGP-C-HA* and a prominent small peptide are immunoprecipitated with the anti-SP7 antibody (Fig. 3*A*, *lane 4*). Both, the 55-kDa protein and the 6-kDa peptide (SP^{GP-C}) are no longer immunoprecipitated when the SP7 peptide is included in the immunoprecipitation (*lanes 7* and *8*). During the 3- and 6-h chase period, the amount of the

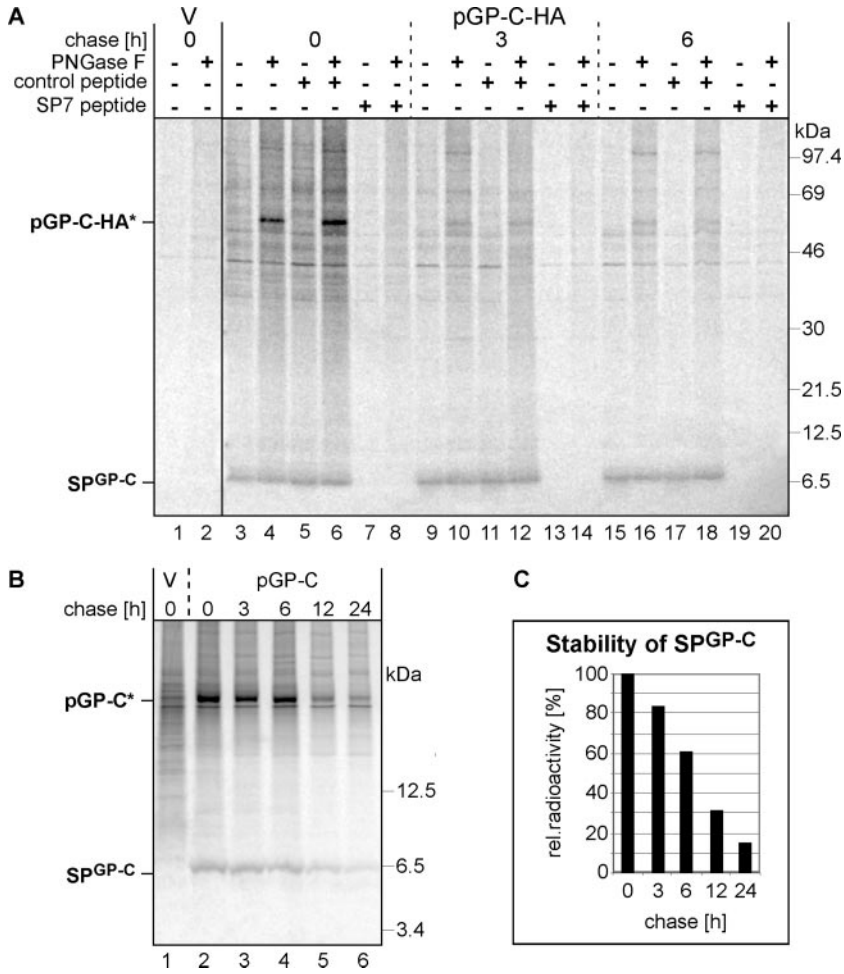

FIG. 3. **Pulse-chase analysis of pGP-C-HA.** In *A*, transfected HeLa cells expressing pGP-C-HA were labeled for 30 min and chased for the times indicated. Solubilized proteins were immunoprecipitated with anti-SP7 antibody and aliquots of these proteins were treated with PNGase F and further analyzed as described in the legend for Fig. 2. The positions of unglycosylated pGP-C-HA* and SP^{GP-C} are shown. *V*, pSV51l. *B*, transfected HeLa cells expressing pGP-C were labeled and immunoprecipitated as described under *A* but chased for 3, 6, 12, and 24 h. Unglycosylated pGP-C* and SP^{GP-C} are indicated. *C*, quantification of the amount of SP^{GP-C} as shown under *B*.

55-kDa protein is substantially reduced, whereas the amount of SP^{GP-C} is not markedly altered (*lanes 4*, *10*, and *16*).

To expand the chase times and see whether the HA tag affects the stability of pGP-C or the SP^{GP-C}, we expressed pGP-C as described above but used chase times of 3, 6, 12, and 24 h. To rule out the possibility that SP^{GP-C} migrates at the gel front, we again used the SDS-PAGE Tricine gel system that separates peptides with high resolution. SP^{GP-C} immunoprecipitated by the anti-SP7 antibody is still detectable after 24 h of chase labeling (Fig. 3*B*). From a quantification of the amount of SP^{GP-C} detectable at the chase times, we calculated a half-life of more than 6 h for SP^{GP-C} (Fig. 3*C*).

Membrane Association and Protease Sensitivity of SP^{GP-C}—To investigate the membrane association of SP^{GP-C}, we pulse-labeled transfected cells and prepared a cytoplasmic and membrane fraction by centrifugation of a cell homogenate. As markers for cytosol and ER membranes, antibodies against α-tubulin and Sec61β were used, respectively (see supplementary data, Fig. 1*S*). SP^{GP-C} was exclusively found in the membrane fraction (Fig. 4, *cf. lanes 2* and *3*). Carbonate (pH 11) extraction was also not able to remove SP^{GP-C} from the membrane fraction (*cf. lanes 10* and *11*). To test whether the SP^{GP-C} was accessible on the cytoplasmic side of the membrane, proteinase K was added to the membrane and cytoplasmic fractions. Neither proteinase K (*lanes 4* and *5*) nor trypsin (data not shown) were able to cleave the SP^{GP-C}. To see whether the resistance against protease digestion was due to the membrane barrier or is an intrinsic property of the SP^{GP-C}, we solubilized the membranes with either the nondenaturing detergent Triton X-100 or increasing concentrations (0.2, 0.5, and 1.0%) of SDS (30 min on ice). Although pGP-C-HA was digested under these conditions, SP^{GP-C} was not (*lanes 6–9*).

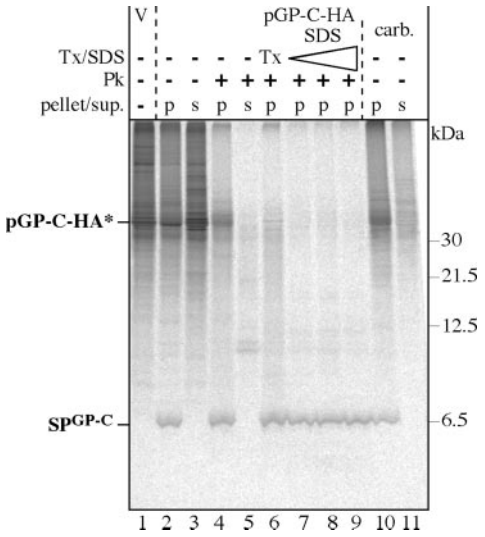

FIG. 4. **Membrane association of SP^{GP-C}.** Transfected pulse-labeled HeLa cells were fractionated into supernatants (*s*) and pellets (*p*) by centrifugation (*lanes 2* and *3*). Aliquots were treated with proteinase K (*Pk*), Triton X-100 (*Tx*), or 0.2, 0.5, or 1% SDS on ice as indicated. Antigens from each fraction were immunoprecipitated with the anti-SP7 antibody. An aliquot of the pellet fraction was extracted with carbonate (pH 11) and separated into supernatant and pellet by centrifugation as indicated (*lanes 10* and *11*). pGP-C-HA* and the SP^{GP-C} are indicated.

SP^{GP-C} after Infection with LCMV—To investigate the cleavage and fate of SP^{GP-C} during viral biogenesis and maturation,

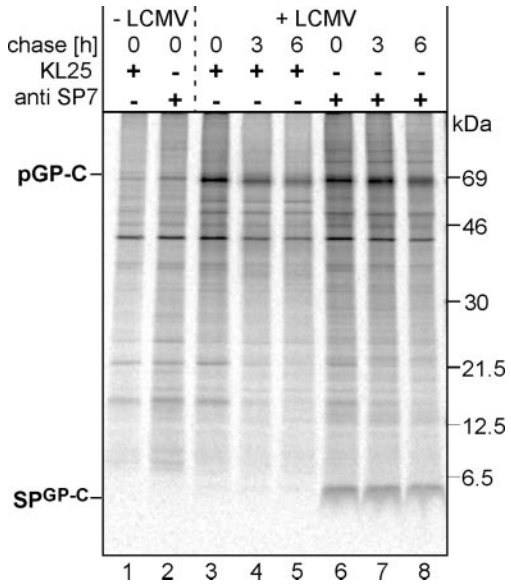

FIG. 5. **Pulse-chase labeling of LCMV-infected cells.** LCMV-infected and -uninfected cells were [^{35}S]Met/Cys-labeled and chased for 3 and 6 h. Antigens were immunoprecipitated with the KL25 antibody recognizing an epitope in the GP-1 protein or with the anti-SP7 antibody and separated on a Laemmli gel. The positions of pGP-C and SP^{GP-C} are indicated.

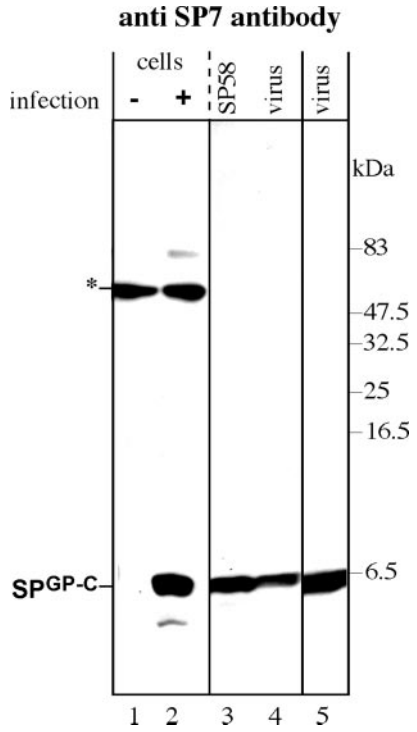

FIG. 6. **Identification of SP^{GP-C} in LCMV particles by Western blot analysis.** Proteins from LCMV-uninfected and -infected cells (*lanes 1* and *2*) and purified virus particles (*lanes 4* and *5*) were separated by SDS-PAGE and probed by Western blotting with anti-SP7 antibody. The position of the SP^{GP-C} is indicated. *, a cellular protein cross-reacting with the anti-SP7 antibody. An *in vitro* synthesized peptide (SP58) comprising amino acid residues 1–58 of SP^{GP-C} is used as a size marker (*lane 3*). In *lane 5*, a longer exposure of *lane 4* is shown.

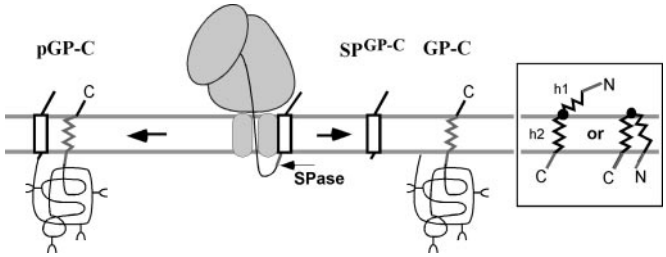

FIG. 7. **Proposed model for signal peptide cleavage from pGP-C.** During membrane insertion, the SP of pGP-C is only cleaved from a subset of synthesized molecules. SP^{GP-C} has a half-life of more than 6 h. It may span the membrane twice as it harbors two hydrophobic regions, h1 and h2 (*boxed inset*), and has a compact structure that is resistant to mild proteolysis even in the presence of detergents. *SPase*, signal peptidase.

we infected MC57 mouse fibroblasts with LCMV. 24 h after infection, cells were labeled with [^{35}S]Met/Cys for 60 min and chased for 3 or 6 h with medium containing nonlabeled amino acids. An antibody recognizing an epitope in the mature GP-1 protein (KL25) and the anti-SP7 antibody were used in immunoprecipitations. As compared with the noninfected cells (Fig. 5, *lanes 1* and *2*), an intensively labeled protein of about 70 kDa was immunoprecipitated with the KL25 antibody as well as the anti-SP7 antibody (*lanes 3–8*). The amount of this protein was reduced at the 3 and 6 h chase times. The size of this protein (70 kDa) and the fact that the protein is immunoprecipitated by KL25 as well as the SP7 antibody suggests that it is mainly pGP-C. Cleaved SP^{GP-C} is clearly detectable in anti-SP7 immunoprecipitates even after 6 h of chase (*lanes 6–8*). Thus the high stability of the SP^{GP-C} is not restricted to transfected cells but is also a property of the SP^{GP-C} during virus infection.

To see whether the cleaved SP^{GP-C} also accumulates in the virus, we purified LCMV particles from culture supernatants of acute infected L929 cells and identified the SP^{GP-C} by using the anti-SP7 antibody and Western blot analysis. As can be seen in Fig. 6, the SP^{GP-C} is detected in the infected cells (*lane 2*) as well as in the purified LCMV particles (*lanes 4* and *5*). The SP^{GP-C} comigrates with *in vitro* synthesized SP^{GP-C} (SP58), indicating that the entire SP accumulates (*cf. lanes 3* and *4*). The purity of the virus particle preparation is indicated by the absence of the cellular 50-kDa protein cross-reacting with the anti-SP7 antibody (*cf.* Fig. 6, *lanes 1* and *2* with *lane 5*). Furthermore, a marker protein for the ER, Sec61β, could not be detected in the LCMV particle preparation (see supplementary data, Fig. 2*S*). In infected cells, the anti-SP7 antibody recognized a peptide smaller than SP^{GP-C} by about 1–2 kDa. This peptide might represent an SPP processing product of the SP^{GP-C} (15).

DISCUSSION

Signal peptides are usually cleaved from their preprotein shortly after membrane insertion and are then rapidly proteolytically processed and/or degraded (3, 6). The SP of LCMV pGP-C is different with respect to cleavage from the preprotein and its processing and degradation (for a schematic representation, see Fig. 7). The SP^{GP-C} is not cleaved from all glycoprotein molecules, and pGP-C accumulates for several hours in transfected and infected cells. More unexpected is that the cleaved SP^{GP-C} is unusually stable, having a half-life of more than 6 h, and accumulates in LCMV particles.

SP^{GP-C} Cleavage from pGP-C—We raised an antibody (anti-SP7) that specifically recognizes the signal peptide of LCMV pGP-C. Specificity was demonstrated by using competing and control peptides in immunoprecipitations. In transfected and LCMV-infected cells, pGP-C and the cleaved signal peptide SP^{GP-C} could be detected and found to be rather long-lived. We were unable to resolve or detect mature GP-C. This may be due to the gel system used or may indicate degradation of GP-C. The recombinant LCMV (WE) pGP-C we used in this study is derived from persistent infected cells. During progression from acute to persistent infection, pGP-C accumulates 12 point mu-

tations (between amino acids 94 and 457), which are exclusively found in the mature part of the glycoprotein but not in its signal peptide (amino acids 1–58). One (L110P) of these point mutations prevents processing into GP-1 and GP-2 (37). However, accumulation of pGP-C and $SP^{GP\text{-}C}$ is also seen in acute infected cells and therefore reflects an intrinsic property of pGP-C and is not due to the mutations accumulating in GP-C in persistent infected cells.

Inefficient SP cleavage has also been found for some other viral membrane proteins. A fraction of newly synthesized HCMV US11 molecules retains the SP after the US11 has been completed (7). Delayed cleavage is caused by amino acid residues in the N-terminal part of the US11 SP. A second region that affects the rate and extent of SP cleavage is the transmembrane region of US11 (7). A precursor form of the HIV-1 gp160 has also been found to accumulate. In this case, the SP cleavage has been shown to be a prerequisite for intracellular transport of gp160. The SP of pre-gp160 interferes with gp160 folding and surface expression (8, 38). Whether similar functions can be assigned to the $SP^{GP\text{-}C}$ awaits further experimentation.

$SP^{GP\text{-}C}$ Accumulation in Infected Cells and in LCMV Particles—In contrast to other SPs, the cleaved $SP^{GP\text{-}C}$ is not rapidly processed and degraded. It accumulates in transfected cells expressing pGP-C as well as in LCMV-infected cells producing virus particles. Stability is thus not dependent on other factors encoded by the viral genome. The basis for the high stability of the $SP^{GP\text{-}C}$ probably lies in the structural features of $SP^{GP\text{-}C}$. Although an average signal peptide comprises about 20 amino acid residues, the $SP^{GP\text{-}C}$ extends over 58 amino acid residues and is characterized by two hydrophobic regions separated by a lysine residue (Fig. 1*B*). The N-terminal h1 region includes 15 uncharged amino acid residues, of which 11 are hydrophobic. The h2 region contains 20 uncharged amino acid residues. Membrane insertion of signal peptides of a secretory or type I membrane protein is thought to occur in a loop-like fashion such that the N terminus is exposed on the cytoplasmic side, and the cleavage site for signal peptidase is exposed on the lumenal side (Fig. 7). Given that the two hydrophobic regions of $SP^{GP\text{-}C}$ are both of sufficient length and hydrophobicity to span the membrane, it is well conceivable that $SP^{GP\text{-}C}$ spans the membrane twice. We have demonstrated using carbonate extraction that all $SP^{GP\text{-}C}$ peptides are integrated in the membrane. As cleavage sites of signal peptidase are usually in proximity to the hydrophobic segment of a signal peptide, we consider it very likely that the h2 region spans the membrane during membrane insertion such that signal peptidase has access to the cleavage site on the lumenal side of the ER. The h1 region is also of sufficient hydrophobicity to span the membrane. If this is true, the N terminus would have to translocate across the membrane, and the h1 and h2 regions would span the membrane in antiparallel configuration (Fig. 7). Several observations argue for such a configuration: 1) Protease added to membrane vesicles does not cleave the $SP^{GP\text{-}C}$. If only the h2 region would span the membrane, the more than 30 amino acid residues of the N terminus would be exposed on the cytoplasmic side accessible to the added protease. 2) The $SP^{GP\text{-}C}$ is very resistant to proteolysis even in the presence of detergents, suggesting a compact structure as formed in the suggested antiparallel configuration of the two h regions. 3) The N-terminal hydrophilic region contains an excess of acidic amino acid residues over basic ones. A statistical analysis has revealed that clusters of positively charged amino acid residues are mostly found on the cytoplasmic side of membrane-spanning proteins (39). As judged by this criterion, the slightly negatively charged N-terminal region of the $SP^{GP\text{-}C}$ would be compatible with translocation across the ER membrane.

An unusually long and stable SP has also been found for the foamy virus envelope glycoprotein (Env) (40). The foamy virus SP^{Env} is 148 amino acid residues in length and contains a single hydrophobic region located between residues 70 and 90. This SP becomes glycosylated and is found in virus particles. What structural features of this SP prevent proteolytic processing and rapid degradation is not yet known.

Possible Implications of $SP^{GP\text{-}C}$ Cleavage and Stability—What relevance could the accumulation of pGP-C and the rather stable $SP^{GP\text{-}C}$ have for the LCMV life cycle? pGP-C might represent a biosynthetic intermediate that could be converted posttranslationally to transport competent GP-C by cleavage of the SP. Alternatively, pGP-C might be a substrate for the ER-associated degradation system. Clearly, further work is required to distinguish between these two or other possibilities.

LCMV is the prototype of the arenaviridae. Glycoproteins of other arenaviridae are synthesized with SPs of similar length. In addition, these SPs show sequence similarity and also contain two hydrophobic segments separated by one or two positively charged amino acid residues (see supplementary data, Fig. 3*S*). Cleavage of Lassa virus signal peptide was recently shown to be necessary for GP-C processing into GP-1 and GP-2 and maturation in the secretory pathway (41).

The unusual stability and accumulation of $SP^{GP\text{-}C}$ strongly argues for functions besides targeting to the ER membrane. An attractive hypothesis would be that $SP^{GP\text{-}C}$ plays a role for virus assembly or formation and stabilization of the virus particle itself. Our finding that $SP^{GP\text{-}C}$ is present in purified virus particles in substantial amounts supports such a notion.

A striking biosynthetic and structural similarity can be found between the $SP^{GP\text{-}C}$ and the 6K peptide of Semliki Forest virus and Sindbis virus (42). The 6K peptides of these viruses are synthesized as part of a polyprotein between the two glycoproteins P62 and E1 and are excised by two proteases to give the final 6K products. The 6K peptides comprise 55–58 amino acid residues and have an acidic, hydrophilic N-terminal region and two hydrophobic segments interrupted by 1 or 2 basic amino acid residues. 6K, like $SP^{GP\text{-}C}$, is thought to be cleaved at its C-terminal side by signal peptidase (42). It is known that Semliki Forest virus 6K is required for efficient virus budding and is found in the virus in submolar amounts (43–47).

A function in virus budding and integration into virus particles has also been shown for the SP of foamy virus Env protein (40). However, this SP does not share many structural similarities with the $SP^{GP\text{-}C}$.

The signal peptide of pGP-C contains the immunodominant MHC class I restricted T cell epitope (gp33) comprising amino acid residues 33–41/43 (23, 24). A quite obvious consequence of $SP^{GP\text{-}C}$ stability is that the T cell epitope will not directly be released, and thus the T cell response will be delayed. Generation of the $SP^{GP\text{-}C}$-derived T cell epitope requires a functional proteasome, suggesting that the $SP^{GP\text{-}C}$ is eventually released from the membrane into the cytosol, where it becomes proteolytically processed (26). Whether processing of cleaved $SP^{GP\text{-}C}$ is relevant for generating the gp33 T cell epitope is unclear. Alternatively, mistargeted pGP-C accumulating during biosynthesis in the cytosol may be degraded by the proteasome and generate the gp33 T cell epitope.

Work described here strongly suggests that the SP of pGP-C is more than a targeting device to the ER. It may function at further stages of the LCMV life cycle.

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