Long-lived Signal Peptide of Lymphocytic Choriomeningitis Virus Glycoprotein pGP-C

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 (LCMV) shows different properties: 1) The SP of pGP-C is unusually long (58 amino acid residues) and contains two hydrophobic segments interrupted by a lysine residue. 2) The SP of pGP-C is cleaved only from a subset of pGP-C proteins. A substantial portion of pGP-C accumulates that still contains the SP. 3) The cleaved SP is rather long-lived (t½ of more than 6 h). 4) The cleaved SP resides in the membrane and is resistant to digestion with proteinase K even in the presence of detergents, suggesting a compact structure. 5) SP accumulates in virus particles. These unusual features of the cleaved SP suggest that SP 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, 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 luminal 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 Ca2+-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 immunoreognition. 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 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 of the SP, is presented by MHC class I molecules to cyto-
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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 pSV511pGP-C was derived from the widely used original cDNA of LCMV (WE) S RNA (27) and the pSV51 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 pSV51 using an additionally introduced BamHI site (pSV511pGP-C).

A DNA fragment encoding pGP-C-HA was generated by polymerase chain reaction using the forward primer 5'-GGAGTCATCTGACGTCAG and the backward primer 3'-CTGAGATCCTCAGTGAAGTCAGAGATGACGACG. The PCR product was inserted into the BamHI site of pSV511 (pSV511pGP-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 of LCMV pGP-C and including amino acid residues 7–18 (MFIMALPHKPV, 31) and 26–41 (TSGIAKYNFY, 32) were synthesized. KL25 is a mouse monoclonal antibody reactive with the LCMV glycoprotein GP-1 (21).

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 x 10^6 confluent cells were starved in Met/Cys-free Dulbecco's modified Eagle's medium plus 10% fetal calf serum for 15 min at 37°C and labeled with 0.15 mCi/ml [35S]Met/Cys for 30 min. The resulting cell lysate was immunoprecipitated with anti-HA or anti-SP7 antibodies using standard Western blot analysis techniques (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. 1A), antibodies against three different segments of the SP^{Gp-C} were raised in rabbits. The antibodies used for immunization comprised amino acid residues 7–18 (SP7), 30–41 (SP30), and 48–57 (SP48) (Fig. 1B). Antibodies affinity-purified and used to immunoprecipitate a fragment of pGP-C, comprising amino acid residues 1–142 (pGP-C_{142}) (Fig. 1A), which was synthesized in vitro. An aliquot of the translation reaction (Fig. 1C, lane 1) and the immunoprecipitates (lanes 2–13) were separated by SDS-PAGE, and pGP-C_{142} was visualized by phosphorimaging. Only the anti-SP7 antibody was able to immunoprecipitate pGP-C_{142} (Fig. 1C, 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-C_{142} (lane 5). Antibodies raised against the other two SP peptides, SP30 and SP48, did not immunoprecipitate pGP-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-C_{142} was synthesized in the presence of rough microsomal membranes (13). Cleavage of the SP^{Gp-C} from pGP-C_{142} was very inefficient as compared with SP cleavage from the secretory protein proprolactin (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. 1A). Cells were labeled with [35S]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 (pGP-C-HA^{+}) was immunoprecipitated with anti-HA or anti-SP7 antibodies (Fig. 2A). Both antibodies immunoprecipitated a major band of about 55 kDa, the expected molecular mass of unglycosylated (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. 2B, 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-
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. 2C, the SPGP-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 SPGP-C—To follow cleavage of the SPGP-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. 3A). 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 SPGP-C. SP7 or control peptides were added to the samples as indicated. During the 3- and 6-h chase period, the amount of the 55-kDa pGP-C-HA* and a prominent small peptide is immunoprecipitated with the anti-SP7 antibody (Fig. 3A, lane 4). Both, the 55-kDa protein and the 6-kDa peptide (SPGP-C) are no longer immunoprecipitated when the SP7 peptide is included in the immunoprecipitation (lanes 7 and 8).
55-kDa protein is substantially reduced, whereas the amount of SPGP-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 SPGP-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 SPGP-C migrates at the gel front, we again used the SDS-PAGE Tricine gel system that separates peptides with high resolution. SPGP-C immunoprecipitated by the anti-SP7 antibody is still detectable after 24 h of chase labeling (Fig. 3B). From a quantification of the amount of SPGP-C detectable at the chase times, we calculated a half-life of more than 6 h for SPGP-C (Fig. 3C).

Membrane Association and Protease Sensitivity of SPGP-C—To investigate the membrane association of SPGP-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 Sec61 were used, respectively (see supplementary data, Fig. 1S). SPGP-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 SPGP-C from the membrane fraction (cf. lanes 10 and 11). To test whether the SPGP-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 SPGP-C. To see whether the resistance against protease digestion was due to the membrane barrier or is an intrinsic property of the SPGP-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, SPGP-C was not (lanes 6–9).

SPGP-C after Infection with LCMV—To investigate the cleavage and fate of SPGP-C during viral biogenesis and maturation,
we infected MC57 mouse fibroblasts with LCMV. 24 h after infection, cells were labeled with [35S]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 SPGP-C is clearly detectable in anti-SP7 immunoprecipitates even after 6 h of chase (lanes 6–8). Thus the high stability of the SPGP-C is not restricted to transfected cells but is also a property of the SPGP-C during virus infection.

To see whether the cleaved SPGP-C also accumulates in the virus, we purified LCMV particles from culture supernatants of acute infected L929 cells and identified the SPGP-C by using the anti-SP7 antibody and Western blot analysis. As can be seen in Fig. 6, the SPGP-C is detected in the infected cells (lane 2) as well as in the purified LCMV particles (lanes 4 and 5). The SPGP-C comigrates with in vitro synthesized SPGP-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. 2S). In infected cells, the anti-SP7 antibody recognized a peptide smaller than SPGP-C by about 1–2 kDa. This peptide might represent an SPP processing product of the SPGP-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 SPGP-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 SPGP-C is unusually stable, having 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.

**Fig. 5.** Pulse-chase labeling of LCMV-infected cells. LCMV-infected and -uninfected cells were [35S]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 SPGP-C are indicated.

**Fig. 6.** Identification of SPGP-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 SPGP-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 SPGP-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. SPGP-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.
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 SPGP-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 SPGP-C awaits further experimentation.

**SPGP-C Accumulation in Infected Cells and in LCMV Particles**—In contrast to other SPs, the cleaved SPGP-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 SPGP-C probably lies in the structural features of SPGP-C. Although an average signal peptide comprises about 20 amino acid residues, the SPGP-C extends over 58 amino acid residues and is characterized by two hydrophobic regions separated by a lysine residue (Fig. 1B). 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 luminal side (Fig. 7). Given that the two hydrophobic regions of SPGP-C are both of sufficient length and hydrophobicity to span the membrane, it is well conceivable that SPGP-C spans the membrane twice. We have demonstrated using carbonate extraction that all SPGP-C peptides are integrated in the membrane. As cleavage sites of signal peptides 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 luminal 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 SPGP-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 SPGP-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 SPGP-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 SPEnv 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 SPGP-C Cleavage and Stability**—What relevance could the accumulation of pGP-C and the rather stable SPGP-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. 3S). 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 SPGP-C strongly argues for functions besides targeting to the ER membrane. An attractive hypothesis would be that SPGP-C plays a role for virus assembly or formation and stabilization of the virus particle itself. Our finding that SPGP-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 SPGP-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 SPGP-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 SPGP-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 SPGP-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 SPGP-C-derived T cell epitope requires a functional proteasome, suggesting that the SPGP-C is eventually released from the membrane into the cytosol, where it becomes proteolytically processed (26). Whether processing of cleaved SPGP-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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