Proteolytic Processing of Filovirus Glycoproteins

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Abstract

Filoviruses (Marburg virus and Ebola virus) have a single envelope glycoprotein (GP) that initiates infection. GP is a class I fusion protein that forms trimeric spikes composed of heterodimers of the subunits GP1 and GP2. GP1 and GP2 are derived from the precursor pre-GP by furin cleavage during exocytosis. GP1 contains a receptor-binding core topped by a glycan cap and a heavily glycosylated mucin-like domain, while GP2 contains a fusion loop and a membrane anchor. After entering cells by macropinocytosis, the glycan cap and the mucin-like domain are removed from GP1 by endosomal cathepsins B and L exposing the binding site for the Niemann-Pick C1 receptor. It appears that there is no strict requirement for specific proteases involved in GP processing. Thus, furin is not indispensible for GP1-2 cleavage, and GP1 may be trimmed not only by cathepsins B and L but also by other endosomal proteases.

Two soluble glycoproteins of Ebola virus are also processed by host proteases. A significant amount of GP1,2 is cleaved by the metalloprotease TACE and shed from the surface of infected cells (GP1,2 delta). The secreted protein sGP is derived from the precursor pre-sGP by furin cleavage.

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5.1 Introduction

Filoviruses comprising Marburg virus (MARV) and 5 Ebola virus (EBOV) species (Zaire, Sudan, Reston, Bundibugyo, and Tai Forest virus) cause fulminant hemorrhagic fevers in man and nonhuman primates. MARV and EBOV have a zoonotic background and, except for Reston virus, are endemic in sub-Saharan Africa. Since the discovery of MARV in 1967 and EBOV in 1976, the viruses re-emerged with increasing frequency. Most of the outbreaks were dramatic but confined to relatively short time periods and small geographic areas. Between 2013 and 2015, however, an unprecedented EBOV outbreak occurred in West Africa with almost 30,000 human infections and more than 11,000 deaths.

The non-segmented negative-stranded RNA genome of filoviruses contains seven genes: NP, VP35, VP40, GP, VP30, VP24, and L. The GP gene of EBOV has two overlapping reading frames from which three glycoproteins are expressed by transcriptional editing: the envelope glycoprotein GP and two nonstructural glycoproteins, sGP and ssGP. In contrast, the envelope glycoprotein of MARV is expressed as the only gene product from a single open reading frame (Volchkov et al. 1995, 2005; Sanchez et al. 1996).

5.2 Biosynthesis and Maturation of Filovirus Glycoproteins

GP is a type I membrane glycoprotein that matures during export through the exocytotic transport route to the cell surface. ER-associated GP, designated pre-GPer, contains oligomannosidic *N*-glycans and shows sensitivity to endoglycosidase H treatment. Oligomerization of GP occurs already within the ER early after pre-GPer synthesis (V. Volchkov, unpublished results). Pre-GPer lacks the signal peptide sequence which is co-translationally cleaved by cellular signal peptidase. The second precursor identified, designated pre-GP, represents the Golgi-associated form of GP. This precursor contains mature *N*-glycans and is O-glycosylated. Still within the Golgi apparatus, pre-GP is processed by proteolytic cleavage into GP1,2 consisting of the amino-terminal fragment GP1 and the carboxy-terminal fragment GP2 linked by a disulfide bond (Volchkov et al. 1998a; Sanchez et al. 1998) (Fig. 5.1). GP1,2 complexes are present at the surface of EBOV-infected cells and build up trimeric spikes on virions. Proteolytic processing of the envelope glycoprotein of filoviruses has been unnoticed for a rather long period of time, largely due to the fact that pre-GP, mature GP1,2, and the GP1 subunit have similar migration rates on polyacrylamide gels and that GP2 tends to escape detection because it partly co-migrates with the VP24 protein. We know now, however, that cleavage of GP is remarkably efficient and that unprocessed GP is not present on Ebola virions in any significant amount.

EBOV GP is cleaved into subunits GP1 and GP2 by furin at the motif R-T-R-R501 (Volchkov et al. 1998a). Furin cleavage was assessed by the observation that cleavage efficiency was dramatically reduced when GP was expressed in the furin-deficient LoVo cell line but was fully restored in these cells by

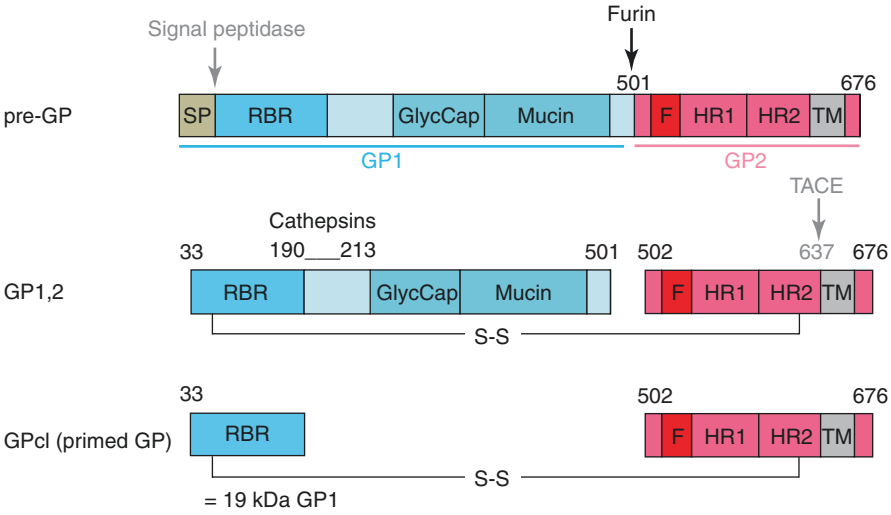

Fig. 5.1 Processing of EBOV GP. GP is a type I membrane protein that matures on the exocytotic transport route. After co-translational removal of the amino-terminal signal by the signal peptidase, pre-GP is cleaved by furin into GP1 and GP2. GP1 contains the receptor-binding region (RBR), the glycan cap (GlycCap), and the mucin-like domain (Mucin). GP2 contains the fusion loop (F), two heptad repeats (HR1, HR2), and the transmembrane anchor. GP1,2 forms trimeric spikes that are incorporated into virions or shed from the cell surface by the metalloprotease TACE. After cell entry by macropinocytosis, glycan cap and mucin-like domain are removed in endosomes by cathepsins yielding GPcl. GPcl contains GP2 linked by a disulfide bond to a 19 kDa fragment of GP1 with the receptor-binding region

vector-expressed furin. The finding that cleavage was effectively inhibited by peptidyl-chloromethylketone containing a furin motif or by site-directed mutagenesis of the furin site further supported this concept.

The surface glycoprotein of MARV is proteolytically processed in a similar way as that of EBOV; two precursor molecules and mature GP1,2 consisting of the disulfide-linked cleavage products GP1 and GP2 were identified in cells expressing MARV GP and in Marburg virions (Volchkov et al. 1998a, 2000). Interestingly, MARV GP contains two sites suitable for furin cleavage: R-R-K-R435 and R-L-R-R561. It appears that the second site is not used for protein processing, possibly due to conformational constraints. Site-directed mutagenesis revealed that MARV GP is indeed proteolytically processed at the first furin site (Volchkov et al. 2000). Mutations introduced at the multibasic site revealed the consensus sequence recognized by furin or the related proprotein convertase PC5/6 which contains Arg at positions −1 and −4 as a minimal requirement and Arg/Lys at position −2 for cleavage optimization (see Chap. 9). Thus, substitution R435L at position −1 resulted in a dramatic loss of cleavage, whereas mutation K434M at position −2 showed a reduction in cleavage efficiency (Volchkov et al. 2000).

A fraction of EBOV GP1,2 that is not incorporated into virions is released from the cell surface after removal of the membrane anchor by the metalloprotease

TACE (tumor necrosis factor-alpha-converting enzyme) (Dolnik et al. 2004) (Fig. 5.1). Released GP1,2, designated GP1,2 delta, is present in the trimeric form which, however, is more labile than GP1,2 trimers, indicating that the membrane anchor has a stabilizing function. GP1,2 delta released from virus-infected cells activates non-infected dendritic cells and macrophages causing the massive secretion of pro- and anti-inflammatory cytokines and increased vascular permeability. These activities may be instrumental for the excessive and dysregulated inflammatory host reactions to infection and, thus, contribute to the high pathogenicity of the virus (Escudero-Pérez et al. 2014). There is also evidence that fine-tuning of the levels of EBOV GP expressed at the surface of infected cells via GP shedding plays an important role in EBOV replication by orchestrating the balance between optimal virion GP content and cytotoxicity caused by GP (Dolnik et al. 2015). TACE, also designated ADAM17, is a member of the ADAM (a disintegrin and metalloprotease) family, a large group of zinc-dependent cell surface proteases. TACE mediates shedding of many membrane proteins and has therefore been proposed to have the function of a common sheddase. Most, but not all, substrates are cleaved between two hydrophobic residues, but neither a specific recognition sequence nor a specific secondary structure at the cleavage site appears to be required (Althoff et al. 2001).

The secreted glycoprotein (sGP) of EBOV is derived from a precursor (pre-sGP) that has a length of 364 amino acids and shares the amino-terminal 295 amino acids with the membrane glycoprotein GP. Like pre-GP, pre-sGP undergoes several co- and posttranslational processing events, such as signal peptide cleavage, N- and O-glycosylation, oligomerization, and proteolytic cleavage by furin to sGP and a small peptide, designated delta-peptide (Volchkova et al. 1998, 1999). sGP, like GP1,2 delta (Dolnik et al. 2004), may have a decoy function by binding EBOV-specific neutralizing antibodies (Sanchez et al. 1996; Volchkov et al. 1998b). There is also evidence that the cytotoxicity caused by GP is down-regulated through the expression of sGP (Volchkov et al. 2001).

5.3 The Role of GP in Host Cell Entry

The mature envelope glycoprotein of filoviruses is a class I fusion protein that forms trimeric spikes composed of disulfide-linked GP1,2 heterodimers. The structure of the EBOV glycoprotein has been analyzed in detail. Early studies gave insight into the post-fusion structure of GP2 (Gallaher 1996; Malashkevich et al. 1999; Weissenhorn et al. 1998a, b; Volchkov et al. 1992). More recently, the structure of GP1,2 trimers in the pre-fusion state has been elucidated (Lee et al. 2008; Lee and Saphire 2009). According to these studies, the trimeric spike is shaped like a chalice. The bowl of the chalice is assembled by the three GP1 subunits, and the base is formed by the GP2 subunits that cradle and encircle the GP1 trimer (Fig. 5.2). The bowl which is formed by discontinuous sections of the amino-terminal region of GP1 (residues 33–226) contains residues required for binding to an endosomal receptor and is covered by a glycan cap with a cluster of N-linked oligosaccharides (residues 227–310). Between the glycan cap and the carboxy-terminal end of GP1

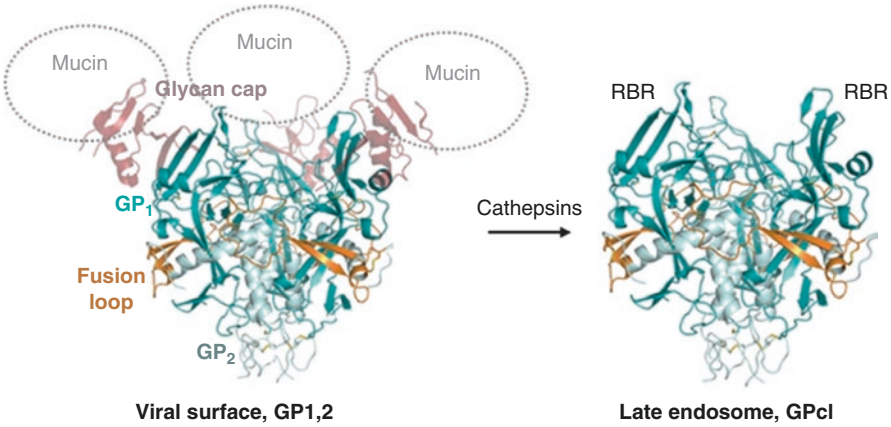

Fig. 5.2 The structure of the EBOV spike. The structure of the trimeric spike before and after removal of glycan caps and mucin-like domains exposing the receptor binding regions (RBR) is shown. GP1 and GP2 are colored in teal and light blue, respectively. Glycan cap (magenta) and fusion loop (yellow) are also indicated. The mucin-like domains were deleted for crystallization and have been modeled here as not-to-scale circles. For crystallization of GPcl, glycan caps have been removed from mucin-deleted GP1,2 by thermolysin treatment (modified from Bornholdt et al. 2016)

stretches a mucin-like domain that is about 150 amino acids long and heavily loaded with *O*-glycans. The GP2 subunit contains the hydrophobic fusion loop, two heptad repeats typical for class I fusion proteins, and the membrane anchor (Fig. 5.1).

GP presumably initiates infection by binding to the cell surface. A number of cell surface receptors have been implicated, but none of them proved to be necessary and sufficient for viral entry. It is widely accepted, however, that filoviruses are internalized after surface attachment by macropinocytosis and transported to endosomes (Saeed et al. 2010; Nanbo et al. 2010; Aleksandrowicz et al. 2011). Within endosomes, EBOV GP1,2 is cleaved by cathepsins B and/or L which is an important step in the infection process (Chandran et al. 2005; Kaletsky et al. 2007; Sanchez 2007; Schornberg et al. 2006). Cathepsin trims EBOV GP1 from its original size (ca. 130 kDa) to an initial 50-kDa fragment, followed by further cleavage to an approximately 19-kDa species of GP1 bound to GP2 by non-covalent linkages and a disulfide bridge between C53 and C609 (Jeffers et al. 2002; Volchkova et al. 1998) (Fig. 5.1). The crystal structure suggests that the site of the final cathepsin cleavage is a loop reaching from residues 189 to 214 (Lee and Saphire 2009). This concept is supported by biochemical studies indicating that the cleavage site is located at amino acid 190 (Dube et al. 2009). Thus, the entire glycan cap and the mucin-like domain are removed yielding a glycoprotein called GPcl that contains the receptor-binding site exposed on the truncated GP1 subunit and the fusion loop on GP2 (Fig. 5.2). The endosomal receptor has been identified as the cholesterol transporter Niemann-Pick C1 (NPC1) (Carette et al. 2011; Côté et al. 2011). NPC1 is a ubiquitously expressed endosomal membrane protein involved in the fusion and fission of endosomes and lysosomes (Goldman and Krise 2010). After cathepsin cleavage and receptor binding, the GP2 subunit unwinds from its GP1 clamp and rearranges irreversibly into a six-helix bundle to drive fusion of viral and

endosomal membrane (Bornholdt et al. 2016;Wang et al. 2016). It has also been suggested that cathepsins are required for a step in genome delivery following fusion triggering (Spence et al. 2016).

Like EBOV, MARV enters cells by macropinocytosis and endosomal fusion, but there are some differences in the structure and in endosomal processing of the glycoproteins. Structural analysis by crystallography and small angle X-ray scattering in solution indicated that the mucin-like domains of EBOV GP project upward, whereas with MARV GP they have a more equatorial orientation. Furthermore, the glycan cap is more flexible with MARV GP than with EBOV GP. Thus, the receptor-binding site appears to be tightly masked on the surface of EBOV spikes but more exposed on the surface of MARV spikes prior to endosomal cleavage (Hashiguchi et al. 2015). This study showed also other structural differences, particularly at the putative cleavage site, which may explain previous observations indicating that, unlike EBOV, MARV does not depend on cathepsin B for endosomal GP processing (Gnirss et al. 2012; Misasi et al. 2012).

5.4 Proteases Responsible for GP Processing

The data presented so far strongly support the concept that removal of the glycan cap and the mucin-like domain which is essential for filovirus infectivity depends on cleavage of GP at the GP1-GP2 interphase followed by endosomal processing of GP1,2 to GPcl. The nature of the proteases responsible for cleavage, however, has been and still is a matter of debate.

The finding that EBOV GP is cleaved into GP1 and GP2 by furin (Volchkov et al. 1998a) did not come as a surprise, since this protease is responsible for the activation of many viral glycoproteins. The role of furin in the EBOV life cycle became a mystery, however, when several groups reported that substitution of all basic amino acids at the furin cleavage site did not significantly affect virus infectivity. Initially, these unexpected data were obtained, when pseudotype systems based on murine leukemia virus (Wool-Lewis and Bates 1999) and vesicular stomatitis virus (Ito et al. 2001) were used which allowed generation of surrogate virions carrying mutated EBOV GP. The mutated glycoprotein was shown to be transported to the plasma membrane and to be incorporated into virions, predominantly in the uncleaved form, and the pseudotyped viruses infected a wide range of cell types from diverse origins. Subsequently, it was reported that recombinant EBOV carrying GP in which the multibasic cleavage site was replaced by nonbasic amino acids was able to replicate in Vero E6 cells (Neumann et al. 2002) and to cause lethal infection in nonhuman primates (Neumann et al. 2007). These findings are frequently used as arguments against an essential function of furin cleavage in EBOV replication. There is evidence, however, that does not fully support this conclusion. Close inspection of the data obtained with the pseudotypes reveals that small amounts of GP1,2 were present. Likewise, a minor, but clearly detectable, fraction of GP was present in the cleaved form in the recombinant EBOV (Volchkov et al. 2005). Furthermore, recombinant EBOV replicated with significantly reduced

growth kinetics, when the furin cleavage site was replaced by nonbasic amino acids (Neumann et al. 2002). It therefore appears that cleavage of GP into subunits GP1 and GP2 is accomplished not only by furin but also, yet with lower efficiency, by other proteases that still have to be identified. It is also conceivable that only a fraction of GP has to be present in cleaved form to allow infection as has been observed with other viruses (see Chap. 6). In any case, there appears to be a preference for furin cleavage, since this is the most efficient processing form. This concept is underlined by the high conservation of the multibasic cleavage site with filoviruses. The only exception is Reston EBOV. Here, the consensus sequence of a typical furin cleavage site is missing which has been suspected to account, at least in part, for the low human pathogenicity of this virus (Volchkov et al. 1998a).

As has been pointed out above, endosomal processing of EBOV GP1,2 is mediated by the cysteine proteases cathepsin B and L. There is evidence, however, that, again, both enzymes are not indispensible for this process. It could be shown that Zaire EBOV entry was reduced in cell culture upon selective inhibition of cathepsin B, but not cathepsin L. Interestingly, all other EBOV species entered the cells efficiently when cathepsin B and/or L activity was blocked. Moreover, cathepsin B and cathepsin L knockout mice were equally susceptible to a lethal dose of mouse-adapted Zaire EBOV as wild-type animals, with no difference in virus replication and time of death (Marzi et al. 2012). Thus, it appears that, like cleavage of GP into subunits GP1 and GP2, endosomal trimming to GPcl is mediated by an array of proteases. This concept is also supported by the observation that cathepsin can be replaced by thermolysin to convert GP1,2 into structurally and functionally competent GPcl (Brecher et al. 2012). EBOV may therefore not be a very suitable target for therapeutic approaches based on protease inhibitors (Marzi et al. 2012), quite in contrast to other viruses, such as influenza virus, where this strategy is more promising because of the high specificity of the proteases required for activation (see Chaps. 8, 9, 11).

Conclusions

Proteolytic processing of the envelope glycoprotein of filoviruses is complex involving a sequence of cleavage steps at different stages of the viral life cycle. In this respect it resembles proteolytic activation of other envelope proteins, such as the F protein of Respiratory Syncytial Virus (see Chap. 2) and presumably the S protein of coronaviruses (see Chap. 4), that are also cleaved first during exocytosis by one and subsequently upon virus entry by another enzyme. Although cleavage of pre-GP to GP1,2 and triming of GP1,2 to GPcl play essential roles in the processing of the filovirus envelope protein, it is not clear whether there is a strict requirement for furin and cathepsins, respectively. The specificity of the cleavage reactions and the proteases involved will have to be analysed in more detail in future studies. It is well known that cleavage primes a viral fusion protein for the conformational change required for activity, but it has never been shown before that fusion activity depends on removal of a large carbohydrate shield from the top of the spike as is the case with filoviruses. Another unique feature is the high amount of virus-encoded glycoproteins that are secreted or shed by proteolytic cleavage from EBOV-infected cells and may play important roles in the course of infection and in pathogenesis.

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