Acta virologica **56**: 283 – 291, 2012 doi:10.4149/av_2012_04_283

Comparative analysis of the envelope glycoproteins of foamy viruses

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Received March 3, 2012; accepted October 19, 2012

Summary. – One of the most fascinating findings in retrovirology is the construction of viral vectors based on foamy viruses (FVs) for gene therapy. The envelope glycoprotein (Env), one of the structural proteins of FV, is an important antigen in the immunoassays, as it is highly specific. To compare the characteristics of all 15 available FV Envs, the phylogenesis, hydrophobicity, modifications, and conserved motifs were analyzed based on the Env sequences. Meanwhile, the secondary structures of transmembrane (TM) domains of FV Envs were predicted. The results of phylogenetic analyses based on Envs indicated that the foamy viruses from different hosts could form three groups. The hydrophobicity analysis revealed that FV Envs had two prominent hydrophobic regions, which was similar to other retroviruses. Though the glycosylation, ubiquitination, and the secondary structures of TM domains of FV Envs were in line with other retroviruses, the roles were distinctly different. Interestingly, the analyses of conserved motifs suggested that FV Envs possessed several specific functional motifs.

Keywords: foamy virus; envelope glycoprotein; sequence analysis; phylogenetic analysis

Introduction

Foamy viruses (FVs) have co-evolved with their natural hosts, including horses, cows, cats, and non-human primates, for an evolutionary history stretching at least 100 million years (Saib, 2003; Switzer *et al.,* 2005). Man is not a natural host for FVs, but can be infected through zoonosis (Heneine *et al.,* 2003). FVs appear to be nonpathogenic in naturally or accidentally infected hosts. This is in contrast to their highly cytopathic properties *in vitro*, where infections result in death of most target cells due to syncytia formation and vacuolization. Owing to their special characteristics, FVs constitute the single genus *Spumavirus* in the subfamily *Spumaretrovirinae* of the family *Retroviridae*, while all the other retroviral genera make up the second subfamily *Orthoretrovirinae*. The typical proviral genome of FVs contains three canonical open reading frames encoding a group-specific antigen (Gag), a polymerase (Pol) and an envelope glycoprotein (Env), flanked by long terminal repeats (LTR). In addition, FVs encode at least two additional non-structural proteins, Tas (transcriptional activator of spumavirus) and Bet. Compared to other retroviruses, FV Env is the only one that interacts specifically with the cognate Gag protein. This interaction is strictly required for targeting of FV capsids to the cellular membrane and for budding of viral particles (Lindemann and Goepfert, 2003). Additionally, FV Env has a highly unusual biosynthesis and

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Abbreviations: BFV = bovine foamy virus; BSV = bovine syncytial virus; EFV = equine foamy virus; Env = envelope glycoprotein; ER = endoplasmic reticulum; ERRS = endoplasmic reticulum retrieval signal; FFV = feline foamy virus; FSV = feline syncytial virus; FP = fusion peptide; FV(s) = foamy virus(es); Gag = group specific antigen; HFV = human foamy virus; LP = leader peptide; MLV = murine leukemia virus; MPMV = Mason-Pfizer monkey virus; MSD = membrane-spanning domain; Pol = polymerase; PSFVaye = aye-aye prosimian foamy virus; SFV_{agm} = African green monkey simian foamy virus; SFV_{cpz} = chimpanzee foamy virus; SFV_{gor} = gorilla foamy virus; SFV_{mac} = macaque foamy virus; SFV_{mar} = marmoset foamy virus; SFV_{orang} = orangutan foamy virus; SFV_{spm} = spider monkey foamy virus; SFV_{sqm} = squirrel monkey foamy virus; SloEFV = sloth endogenous foamy virus; SP = signal peptide; SU = central surface; TM = transmembrane

maturation pathway. It is translated as a full-length precursor protein into the rough endoplasmic reticulum (ER) (Luftenegger *et al.,* 2005). During the transport to the cell surface, the precursor is post-translationally cleaved into three subunits by cellular proteases, which are most likely furin or furin-like proteases, instead of the signal peptidase complex (Duda *et al.,* 2004; Geiselhart *et al.,* 2004).

To better understand the features of FV Envs for developing the FV vectors, the characteristics of FV Envs had been analyzed *in sillico* using all 15 available FV Env sequences. In parallel, the comparison of FV Envs and several other retrovirus Env proteins had been performed.

Materials and Methods

The sequences analyzed in this article were obtained by searching the NCBI Nucleotide database and Protein database. The Env sequence of sloth endogenous foamy virus (SloEFV) was deduced from a previously published paper (Katzourakis *et al.,* 2009). The alignments of multiple sequences were analyzed using the ClustalW2 program (http://www.ebi.ac.uk/clustalw2). Phylogenetic analysis was performed with the maximum likelihood method (MEGA 4.1 Beta), and bootstrap resampling with 1,000 replicates was employed to place approximate confidence limits on individual branches. The hydrophobicity analysis was made with the ExPASy Proteomics Server using the scale Hphob./Kyte & Doolittle (http://web.expasy.org/cgi-bin/protscale/protscale.pl). The putative N-glycosylation sites were predicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc) and the sequon finder 1.0 (http://www.functionalglycomics.com.cn/ourworks/bioinf/bioinf.htm). The prediction of the signal peptide cleavage sites was made by the SignalP program (http://www.cbs.dtu.dk/services/SignalP-4.0/). The prediction of the secondary structure of the transmembrane domain (TM) was made using Secondary Structure Prediction server – PHD (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_phd.html).

Table 1. Foamy viruses analyzed in this study

Acc. No. of complete genome sequence	Virus	Host species
NC_001831	BFV	Bovine
U94514	BSV	Bovine
AF201902	EFV	Equine
NC_001871	FFV	Feline
U85043	FSV	Feline
U21247	HFV	Human
NC_010820	SFV_{agm}	African green monkey
U04327	SFV_{cpz}	Chimpanzees
HM245790	SFV_{gor}	Gorilla
NC_010819	SFV_{mac}	Macaque
GU356395	SFV_{mar}	Marmoset
AJ544579	SFV_{orang}	Orangutan
EU010385	SFV_{spm}	Spider monkey
GU356394	SFV_{sqm}	Squirrel monkey
	SloEFV[a]	Sloth

[a]The sequence of SloEFV was cited from the reference (Katzourakis *et al.,* 2009).

Results

Phylogenetic analysis of FVs based on envelope glycoproteins

Till now, the complete genome sequences of 15 FVs were reported from 9 primate and 6 non-primate hosts (Table 1). To shed light on the evolutionary connections among these viruses, the Env sequences of 15 FVs were used to perform phylogenetic analyses. According to the UPGMA tree (Fig. 1a), the 15 sequences formed 3 different monophyletic groups. The results revealed that all the primate isolates clustered together, while the non-primate isolates formed the second cluster. Meanwhile, SloEFV grouped in the third group, independently.

The phylogenetic analyses of FV Pol and Gag protein sequences were performed in the same way. Comparing the three phylogenetic trees, the phylogenetic tree based on Pol (Fig. 1b) was identical to the one based on Env. But a distinct difference in the grouping of SloEFV could be observed in the phylogenetic tree based on Gag (Fig. 1c).

Hydrophobic regions in the envelope glycoproteins of FVs

All of the 15 Envs possessed the same general structure, which comprised the N-terminal leader peptide (LP) domain, the central surface (SU) domain, as well as the C-terminal transmembrane (TM) domain. Human foamy virus (HFV) Env was revealed to have two prominent hydrophobic regions according to the hydrophobicity analysis (Fig. 2). One was located in the LP domain close to the signal peptide cleavage site, and the other was in the C-terminus of the TM domain. Similarly, there were also two hydrophobic regions shown in all other 14 FV Envs by further analyses. In line with the HFV Env, the two hydrophobic regions were located in the same regions. In the center of the LP domain was a long hydrophobic domain (h-domain) containing 6 to 15 aa. In addition, in the C-terminus of the TM domain, a cluster of several evolutionary conserved amino acids, named membrane-spanning domain (MSD), was found in all 15 FV Envs.

N-linked glycosylation of the envelope glycoproteins of FVs

Before reaching the mature functional state, FV Envs go through a series of modifications, among which the addition

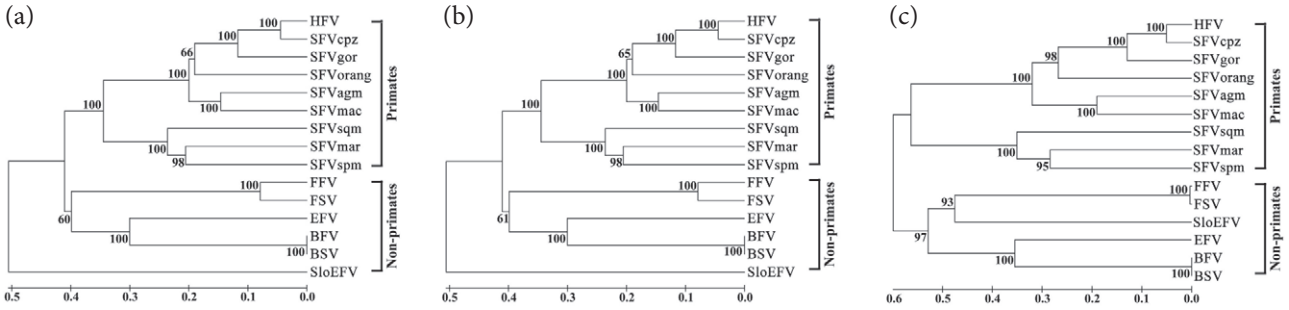

Fig. 1

The phylogenetic analysis of structural proteins of FVs

Phylogenetic analyses based on sequences of Env (a), Pol (b), and Gag (c).

of N-linked glycosylation is indispensable. The glycosylation site of covalent attachment is the asparagine residue (N) within the canonical amino acid sequence, asparagine-X-serine/threonine, where X could be any amino acid except proline, which is avoided due to conformational hindrance (Rao and Wollenweber, 2010). The analyses show that there are 15 potential N-glycosylation sites in HFV Env, 2 in the LP domain (N_{25} and N_{109}), 10 in the SU domain (N_{141}, N_{183}, N_{286}, N_{311}, N_{346}, N_{391}, N_{405}, N_{423}, N_{527} and N_{556}), and 3 in the TM domain (N_{782}, N_{808} and N_{833}) (Fig. 3). However, only 14 N-glycosylation sites in HFV Env were experimentally verified, because N_{25} is located intracytoplasmically and thus the N-glycosylation is inaccessible (Duda *et al.,* 2004).

The potential N-glycosylation sites in the Envs of other 14 FVs were identified using the same programs, the NetNGlyc 1.0 and the sequon finder 1.0. The results indicated that the N-glycosylation sites are located at similar positions, but the number of the sites range from 12 to 16 in the different FV Envs (Fig. 3). The results also revealed that 2 sites in the SU domain (N_{391} and N_{556} in HFV) and 3 sites in the TM domain (N_{782}, N_{808} and N_{833} in HFV) were conserved.

Furthermore, the potential N-glycosylation sites were predicted in human immunodeficiency virus 1 (HIV-1) and murine leukemia virus (MLV), which belong to *Orthoretrovirinae*. The Env of HIV-1 had approximately 30 N-glycosylation sites in the SU and TM domains, whereas the Env of MLV harbored 8 sites in the SU domain and 1 in the TM domain (data not shown). The results demonstrated that FV Envs have similar N-glycosylation sites and numbers, which are distinctly different from other retroviruses.

Ubiquitination of the envelope glycoproteins of FVs

Another important modification of Env is ubiquitination. Usually, ubiquitin is covalently linked to proteins by attachment to the ε-amino group of the lysine residue. It was found that for HFV Env, there are 5 potential ubiquitination

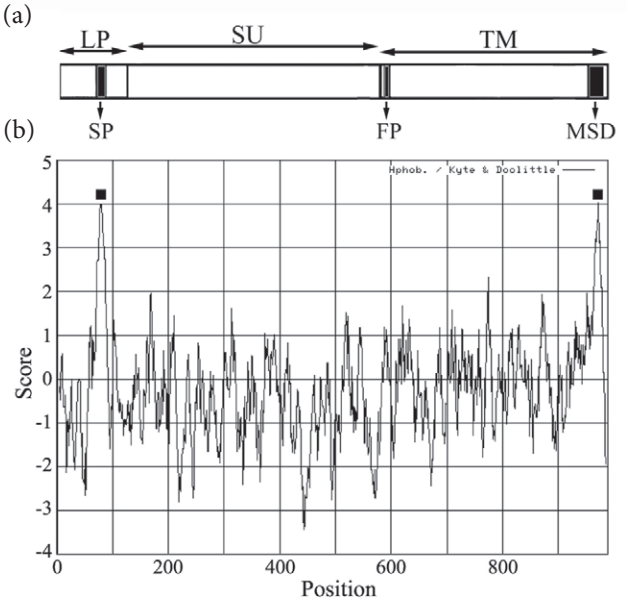

Fig. 2

The hydrophobicity analysis of the envelope glycoprotein precursor of HFV

Individual domains of the precursor (a), hydrophobicity analysis (b).

sites, K_{14}, K_{15}, K_{18}, K_{34}, and K_{53} in the LP domain, which is located in the N-terminal cytoplasmic region (Stanke *et al.,* 2005) (Fig. 4).

Further analyses showed that the potentially ubiquitinated lysine residues were not limited to HFV, but were common to other FV Envs. Except in SFV_{gor} and SFV_{orang} Envs, the ubiquitination sites were relatively conserved in the primate isolates and could be divided into 4 regions, labeled as A, B, C, and D in the figure (Fig. 4). The ubiquitination sites in the regions A, B, and C are crucial for FV particles release (Stanke *et al.,* 2005). However, the conserved ubiquitination region D, which might have

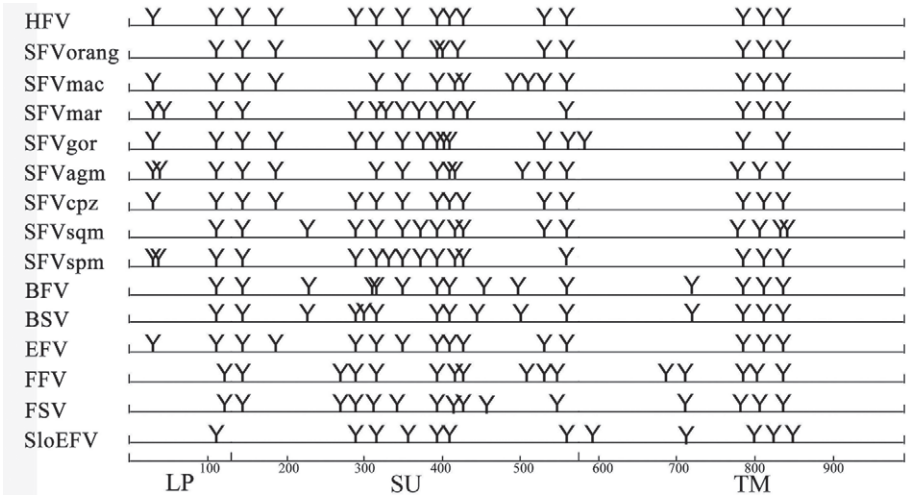

Fig. 3

N-glycosylation sites in the envelope glycoprotein precursors of FVs

The predicted sites are marked with Y.

Fig. 4

Ubiquitination sites in LP domains in the envelope glycoprotein precursors of FVs

The sites labeled as A-D are boxed.

certain important functions, has not been investigated. In non-primate FVs, the ubiquitination sites and numbers of FV Envs varied according to their hosts. In contrast, SloEFV had unique ubiquitination sites.

Conserved motifs in envelope glycoproteins of FVs

All primate FV Envs, except those of SFV_{gor} and SFV_{mar}, had an identical potential signal peptide cleavage site between cysteine and phenylalanine (Fig. 5). In the 6 non-primate FV Envs, the sites varied remarkably. It was between alanine and valine in bovine foamy virus (BFV) and bovine syncytial virus (BSV) Envs, between serine and threonine in feline foamy virus (FFV) and feline syncytial virus (FSV) Envs, between alanine and leucine in EFV Envs, and between glycine and phenylalanine in SloEFV Env.

For the FVs budding process, at least two essential interactions between Env and Gag are required. In Env, the two interaction sites included the MSD in the C-terminal region of TM domain, and the conserved WXXW motif in the N-terminal cytoplasmic region of LP domain (Lindemann *et al.,* 2001; Pietschmann *et al.,* 2000). According to our in-

vestigation, the WXXW motif was identified in LP domain of all 15 Envs (Fig. 5). This finding confirmed the hypothesis that the interaction between FV Env and Gag was required for budding of FV particles.

Besides the WXXW motif, some other conserved motifs were identified in FV Envs. For example, at least 1 RXXR motif was identified in all FV Envs. In some cases, such as SFV_{orang} and FFV, 2 RXXR motifs were found to overlap in analogous regions. These conserved RXXR furin-like protease cleavage sites were located at the positions appropriate to cleave FV Env into their mature LP, SU, and TM domains (Fig. 5), suggesting that all FV Envs displayed similar LP/SU/TM processing mediated by furin or furin-like cellular proteases (Duda *et al.,* 2004). The cleavage mechanism of SloEFV Env is not clear, since it includes only 1 RXXR motif. Furthermore, the Env precursors in other retroviruses are considered to be processed by two different cellular proteases. The first cleavage occurring in ER removes the signal peptide (SP) by cellular signal peptidases. In analogy to other FVs, the second cleavage, which separates SU and TM domains, is considered to be performed by furin or furin-like cellular proteases (Geiselhart *et al.,* 2004).

A highly conserved segment downstream from the RXXR cleavage sites was identified in all FV Envs. It has been predicted to be a fusion peptide (FP) with a typical i, i+3/4, and i+7 pattern. Thus, the FP tends to form an amphipathic α-helix structure (Callebaut *et al.,* 1997; Durell *et al.,* 1997). The function of the FP is still unclear. But it has been proven that the FPs in almost all FVs need to be exposed to a low pH, in order to insert themselves into the target membranes (Picard-Maureau *et al.,* 2003).

The conserved endoplasmic reticulum retrieval signal (ERRS), also named as the dilysine motif (lysine at -3, lysine or arginine at -4 or/and -5 relative to the C-terminus), was found in the TM domains of 14 FV Envs except EFV (Fig. 5). Virion budding of most FVs was observed predominantly at endomembrane (e.g., ER, Golgi), but, to a limited extent, also at the plasma membrane (Lindemann and Rethwilm, 2011). It is the ERRS located in the cytoplasmic region of Env TM domain that plays a critical role in FVs budding from the ER. In EFV, the lack of the functional ERRS motif led to budding of EFV from the plasma membrane rather than from the ER.

Secondary structures of transmembrane domains in the envelope glycoproteins of FVs

The possible secondary structures of the TM domains of all FV Envs were predicted. The data revealed three structural regions within the TM domain: two long α-helix regions at the N-terminus and the C-terminus, and a long alternating region of extended beta-sheets and coils in the middle (Fig. 6). The PHD server was employed to predict the TM domain of HIV-1, Mason-Pfizer monkey virus (MPMV) and MLV, respectively. Similar to the FV TM domain, the α-helix region could be divided into two major parts, one at the N-terminus and the other at the C-terminus. Between the two α-helix regions, there was also a central region of beta-sheets and coils (Fig. 7). As the percentage of α-helix could reach up to more than 60% in HIV-1, MPMV and MLV, the α-helix region was longer while the central region was much shorter than that in FVs

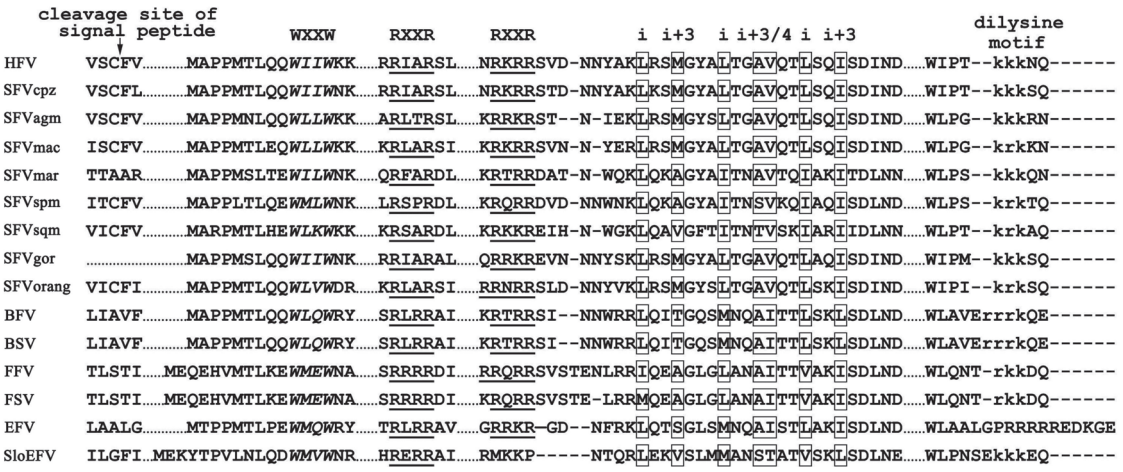

Fig. 5

Conserved motifs in the envelope glycoprotein precursors of FVs

A partial sequence with cleavage sites of signal peptide (arrow), the WXXW motif (italicized), the furin or furin-like cleavage sites RXXR (underlined), the i, i+3, i+3/4 pattern (boxed) in the FP subdomain and the dilysine motif (in small letters) in TM domain.

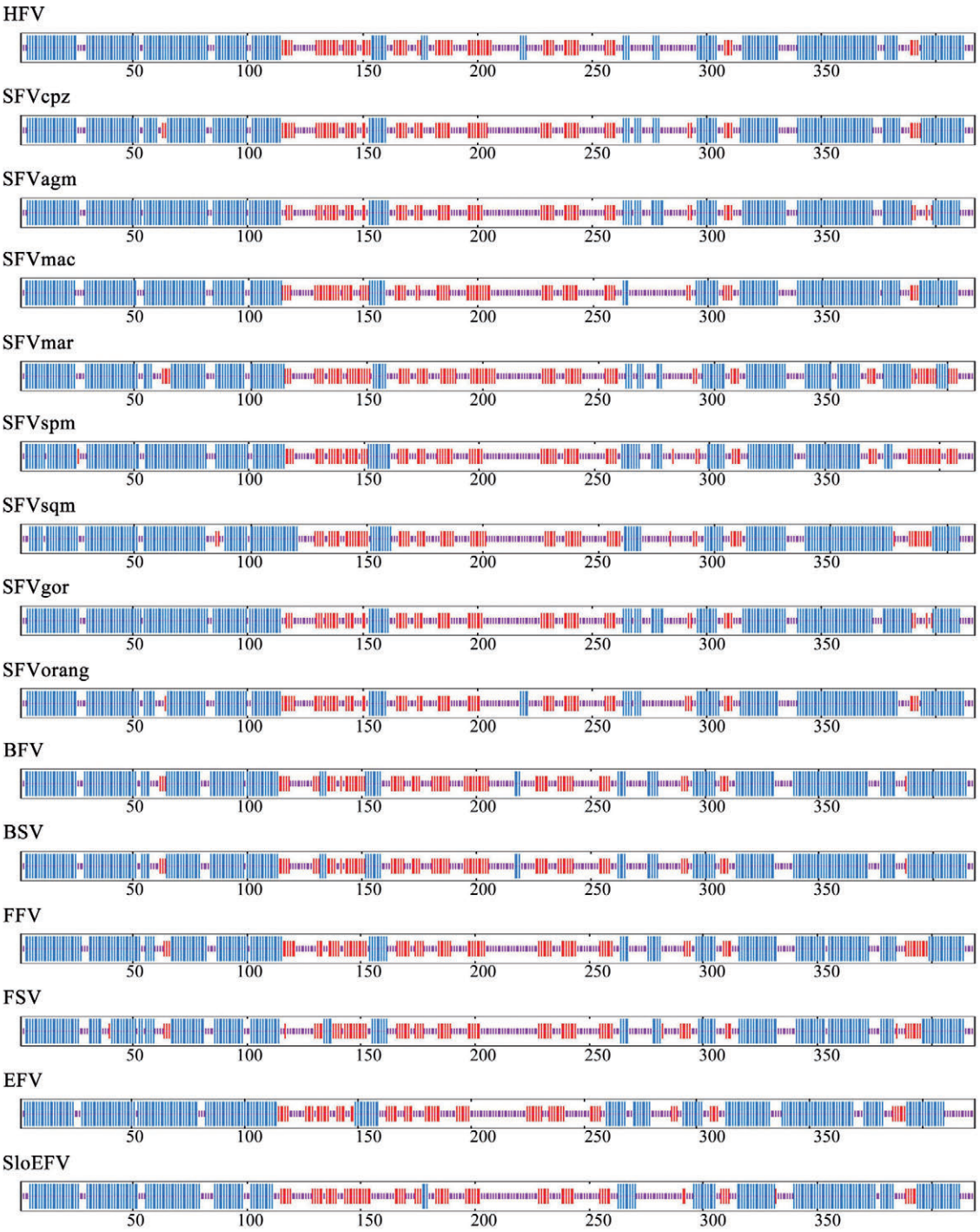

Fig. 6

Secondary structure of TM domains in the envelope glycoprotein precursors of FVs

α-helix (long vertical bar), β-sheet (middle vertical bar), random coil (short vertical bar).

(Table 2). Though the biological functions of the distinctive structure of the TM domain are still unclear, the long central region increases the length of the extracellular domain of FV TM domain, which is responsible for FV

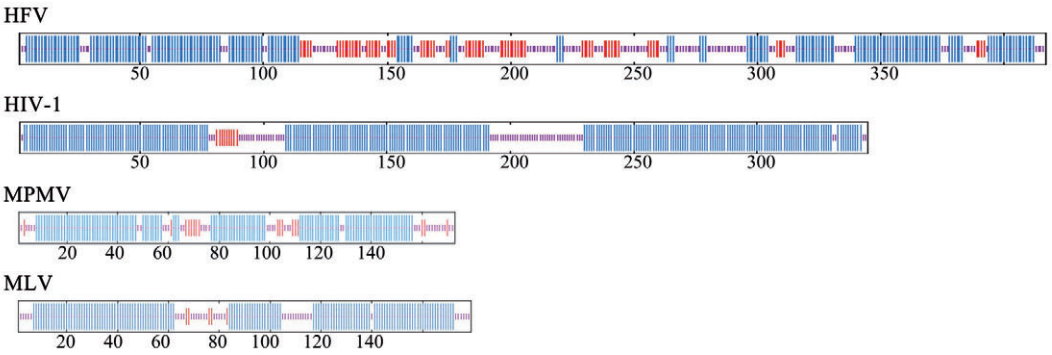

Fig. 7

Secondary structure of TM domains in the envelope glycoprotein precursors of some retroviruses

α-helix (long vertical bar), β-sheet (middle vertical bar), random coil (short vertical bar).

Env appearance as very prominent, long, regularly spaced spike under electron microscope.

In addition, seven to eight conserved cysteine residues have been found in the long central region of FV TM domain. In contrast, only two or three cysteine residues have been identified in HIV-1, MPMV, and MLV in the equivalent region.

Discussion

FVs possess several unique structures and biological characteristics, which set FVs apart from all the other retroviruses. In this paper, the Env sequences of all available 15 FVs isolated from different hosts were analyzed to uncover the characteristics of FV Envs. In the phylogenetic analyses, the tree based on Env was similar to the tree reported previously though only 7 Envs were used at that time (Wang and Mulligan, 1999). The tree based on Pol was exactly the same as Env, while the grouping of SloEFV was different in the tree based on Gag. SloEFV is an ancient foamy virus identified from the two-toed sloth (*Choloepus hoffmanni*), which is geographically and genetically isolated. Thus, the genome of SloEFV is special. The phylogenetic results based on Env and Pol could separate SloEFV from other foamy viruses. Thus, it was accurate to use Env or Pol, but not Gag, for the phylogenetic analysis of FVs.

When this paper was in preparation, a new endogenous foamy virus (aye-aye prosimian foamy virus, PSFVaye) has been identified within the genome of the aye-aye (*Daubentonia madagascariensis*) (Han and Worobey, 2012). However, the reported PSFVaye Env, Pol and Gag are incomplete. Thus, the phylogenetic analyses were carried out with the partial conserved sequences of PSFVaye Env, Pol, and Gag, and the corresponding sequences of the other 15 FVs, respectively. The tree based on Env was exactly the same as Pol, while the grouping of SloEFV was different in the tree based on Gag (data not shown). The results were identical with the previous phylogenetic analyses based on full length of Env, Pol, and Gag, which supported the hypothesis that Env or Pol was appropriate for phylogenetic analysis of FVs. At the same time, all trees, no matter based on Env, Pol or Gag, indicated that PSFVaye was divergent from all the other FVs. These results were the same as the published data.

Table 2. Secondary structure of TM domain of foamy viruses and several other retroviruses

Virus	α-helix (%)	β-sheet (%)	Random coil (%)
BFV	50.00	19.14	30.86
BSV	50.00	19.14	30.86
EFV	52.01	17.73	30.26
FFV	46.30	20.76	32.94
FSV	44.87	19.33	35.80
HFV	48.92	18.47	32.61
HIV-1	77.79	2.61	19.42
MLV	73.74	2.79	23.46
MPMV	67.05	9.83	23.12
SFV_{agm}	50.84	16.31	32.85
SFV_{cpz}	47.72	18.71	33.57
SFV_{gor}	50.84	16.31	32.85
SFV_{mac}	47.60	18.03	34.38
SFV_{mar}	41.99	23.79	34.22
SFV_{orang}	48.68	17.03	34.29
SFV_{spm}	43.13	23.13	33.73
SFV_{sqm}	46.84	19.42	33.74
SloEFV	45.58	18.14	36.28

The hydrophobicity analysis indicated that two hydrophobic regions existed in FV Envs. This coincided with the fact that FV Envs were type III trans-membrane proteins, anchored two times in the cellular membrane, one time at the N-terminal h-domain in LP domain, and the other time at the C-terminal MSD in the TM domain. The interaction between the h-domain and the lipid membrane is essential for the budding of the FV particles. The MSD has been reported to be sufficient for membrane anchorage and further promotion of FV particles release (Pietschmann *et al.,* 2000).

For retroviruses, the carbohydrates of their Envs have been reported to play important roles in the whole viral replication, such as proper folding of Envs, assembly of immunodominant epitopes or regulation of co-receptor usage (Schonning *et al.,* 1996; Reitter and Desrosiers, 1998; Polzer *et al.,* 2001, 2002). While the FV Envs are unique, their N-glycosylation, especially the N-glycosylation at conserved sites, serves several specific functions other than the typical functions of the retroviral Envs. First, the N-glycosylation sites around aa 390 in the SU domain and around aa 780 and aa 830 in the TM domain have influence on viral particle release and transport (Luftenegger *et al.,* 2005). Second, the role of N-glycosylation at N_{109} – the first glycosylation site in the SU domain – in HFV has been identified to interact with the LP domain cleavage, which is essential for HFV particle release and infectivity (Luftenegger *et al.,* 2005). Whether the first N-glycosylation site in the SU domain has the same function in other FV species is still open to investigation. Third, the sequences surrounding the last N-glycosylation site in Envs might contribute to FVs′ temperature-sensitive phenotype (Chan *et al.,* 1997). From the above it can be concluded that the N-glycosylation, particularly at the conserved sites, plays a critical role in FV particle release and budding.

The ubiquitin system mainly participates in the pathway of protein degradation. For example, ubiquitination of HIV-1 Env results in detainment of the protein in the ER, and the degradation of HIV-1 Env might be carried out. However, the ubiquitination of HFV Env results in completely different events, such as additional release of capsidless subviral particles from infected cells (Lochelt *et al.,* 1993; Chan *et al.,* 1997; Goepfert *et al.,* 1997). Possible functions of ubiquitination of Envs of other FVs have not been investigated until now. According to our analyses, the ubiquitination sites and numbers varied in different FV Envs. Thus, the ubiquitination of Envs of other FVs might have variant functions.

To summarize, FV Envs possess many distinctive traits, which are necessary in FVs replication. Especially, these specific features make FV Envs play a unique role in viral particle budding and release. In addition, FV Envs can not be replaced by heterologous viral glycoproteins. The results of our work made a detailed illustration of the structures and functions of FV Envs. More importantly, it might be helpful in the improving the FV vector construction.

Acknowledgements. This work was supported by the grant No. 108184 from the Key Project of Chinese Ministry of Education and the grants No. 31000086, No. 31170154 from the National Natural Science Foundation of China.

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