Virology 498 (2016) 250–256

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The *Lyssavirus* glycoprotein: A key to cross-immunity

Sindisiwe G. Buthelezi [a,b], Heini W. Dirr [b], Ereck Chakauya [a], Rachel Chikwamba [a], Lennart Martens [c,d], Tsepo L. Tsekoa [a], Stoyan H. Stoychev [a,*], Elien Vandermarliere [c,d,e]

[a] Council for Scientific and Industrial Research, Biosciences Unit, Pretoria, South Africa
[b] Protein Structure-Function Research Unit, School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa
[c] Unit for Computational Omics and Systems Biology, Medical Biotechnology Center, VIB, Ghent, Belgium
[d] Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium
[e] Bioinformatics Institute Gent, Ghent University, Ghent, Belgium

ARTICLE INFO

Article history:
Received 21 July 2016
Returned to author for revisions
22 August 2016
Accepted 30 August 2016

Keywords:
Rabies
Lyssavirus
Glycoprotein
Cross-neutralization

ABSTRACT

Rabies is an acute viral encephalomyelitis in warm-blooded vertebrates, caused by viruses belonging to *Rhabdovirus* family and genus *Lyssavirus*. Although rabies is categorised as a neglected disease, the rabies virus (RABV) is the most studied amongst *Lyssaviruses* which show nearly identical infection patterns. In efforts to improving post-exposure prophylaxis, several anti-rabies monoclonal antibodies (mAbs) targeting the glycoprotein (G protein) sites I, II, III and G5 have been characterized. To explore cross-neutralization capacity of available mAbs and discover new possible B-cell epitopes, we have analyzed all available glycoprotein sequences from *Lyssaviruses* with a focus on sequence variation and conservation. This information was mapped on the structure of a representative G protein. We proposed several possible cross-neutralizing B-cell epitopes (GUVTTTF, WLRTV, REECLD and EHLVVEEL) in complement to the already well-characterized antigenic sites. The research could facilitate development of novel cross-reactive mAbs against RABV and even more broad, against possibly all *Lyssavirus* members.

1. Background

Rabies virus (RABV), the causative agent of the rabies disease, belongs to the *Rhabdovirus* family and genus *Lyssavirus*. Rabies is still classified as a neglected tropical disease (WHO, 2013), although the first suggested description of a possible rabies infection dates back to the twenty-third century BC (John, 1997). This zoonosis remains a problem today especially in developing countries of Africa and Asia (WHO, 2005). However because *Lyssaviruses* are capable of infecting most mammalian orders, they remain a threat to the developed world as well (Velasco-Villa et al., 2008), with bats as global reservoirs (Rupprecht et al., 2002).

After introduction into the body through a bite, virions access to and enter nerve endings. The virus travels to the central nervous system and spreads within the brain (John, 1997). At this phase, symptoms such as difficulty in swallowing, excessive salivation, weakness, paralysis and seizures appear (Yousaf et al., 2012). From the brain the virus replicates and continues to spread *via* peripheral nerves to many tissues, including salivary and adrenal glands, skeletal and myocardial muscles and skin (John, 1997). To prevent death in humans the bite wound must be thoroughly cleaned shortly after exposure (WHO, 2005), followed by post-exposure prophylaxis (PEP). Modern PEP includes the administration of passive immunization with anti-rabies immunoglobulin (RIG) and active immunization with rabies vaccine. Human (HRIG) and equine (ERIG) polyclonal anti-rabies immunoglobulins are currently used for passive immunization (WHO, 1997; Yousaf et al., 2012). These RIGs target the viral G protein which is involved in receptor recognition, virion attachment and fusion with the host cell. The G protein is also an essential component in the immunogenic response developed against the *Lyssaviruses* (Müller et al., 2009; Kuzmina et al., 2013).

The use of RIG in PEP poses several problems, as they are prepared from pooled sera obtained from human or horses. There are safety concerns, such as serum sickness or anaphylactic shock, as well as the potential risk of contamination by unknown agents and pathogens (Champion et al., 2000). Moreover, because HRIG originates from hyper-immunised humans, it is only available in limited quantities (Müller et al., 2009). Research efforts have been focused towards the identification of affordable, safe and effective alternative treatments. One alternative is the use of monoclonal antibodies (mAbs) that originate from mice (Müller et al., 2009) or

Abbreviations: RABV, Rabies virus; G protein, glycoprotein; PEP, Post-exposure prophylaxis; RIG, Anti-rabies immunoglobulin; HRIG, Human anti-rabies immunoglobulins; ERIG, Equine anti-rabies immunoglobulins; mAbs, Monoclonal antibodies; WHO, World Health Organization; VSV, Vesicular stomatitis virus; ASA, Accessible surface area; RSA, Relative solvent accessibility

* Corresponding author.
E-mail address: Sstoychev@csir.co.za (S.H. Stoychev).

http://dx.doi.org/10.1016/j.virol.2016.08.034
0042-6822/

that are expressed in plants such as the *Nicotiana benthamiana* tobacco plant (Tsekoa et al.). Nanobodies, which are the functional antigen-binding fragments of camelid heavy chain-only antibodies have also been successfully explored as alternatives for RIG (Terryn et al., 2014).

Because there are several characterized mAbs that have been shown to neutralize the *Lyssaviruses* both *in vitro* and *in vivo* (Kuzmina et al., 2013), the World Health Organization (WHO) Collaborating Centers for Rabies Research have proposed PEP before signs based on a cocktail composed of some of these mAbs. For the cocktail to be effective, the mAbs should target distinct epitopes and should preferably not compete for binding to the G protein. Moreover, such mAbs should address the natural variation among rabies virus field isolates. Virus variants that are susceptible to one mAb might be resistant to the other mAb and *vice versa* (Marissen et al., 2005).

Moreover, besides RABV, some of these mAbs should also neutralize other members of the *Lyssavirus* genus (Terryn et al., 2014). To broaden their use in human PEP, mAb reactivity should neutralize all RABVs and other rabies-related *Lyssaviruses* (Jallet et al., 1999) because new *Lyssaviruses* continue to emerge in various regions world-wide (Kuzmin et al., 2003).

2. Methods

In the current study, we explored the cross-neutralization capacity of several mAbs among the different *Lyssaviruses*, and thoroughly analyzed all available G protein sequences from *Lyssaviruses* with a focus on sequence variation, conservation and the associated sequence entropy. This information was mapped on the structure of a representative G protein which allowed us to propose several possible cross-neutralizing B-cell epitopes of all *Lyssaviruses*.

2.1. Retrieval of sequences

All the *Lyssavirus* G protein sequences were retrieved (retrieval date: 06-2016) from UniProtKB (UniProt Consortium, 2015) based on their taxon identification (TaxonID 11286) (5466 sequences). Non-G proteins and G protein fragments were removed from the list with the aid of scripts written in Python (Python Software Foundation. Python Language version 2.7) (see Supplementary Material 1).

After the filtering process, a total of 1218 sequences were left and used for further analysis (Supplementary Material 2). Of these sequences, 1139 glycoprotein sequences are from the rabies virus. The consensus sequence of the rabies virus G protein sequences and all *Lyssavirus* G protein sequences was calculated with the aid of cons which is part of EMBOSS (Rice et al., 2000).

2.2. Identification of the conserved regions

Scop3D (Vermeire et al., 2015) was used to calculate the sequence variation and entropy of the G protein sequences both from all the *Lyssavirus* members and from all rabies virus G protein sequences. The vesicular stomatitis virus (VSV) glycoprotein (PDB-entry 5i2s (Roche et al., 2006)) was used to visualize the sequence conservation and entropy. Figures were created with PyMOL (PyMOL).

2.3. Calculation of the solvent accessibility

DSSP (Kabsch and Sander, 1983) was used to calculate the solvent accessible surface area (ASA) (Touw et al., 2015). The relative solvent accessibility (RSA) was subsequently obtained relative to the ASA of the residue in a Gly-X-Gly surrounding (Samanta et al., 2002). According to Rost and Sander (Rost and Sander, 1994) residues with RSA of $>15\%$ are considered to be exposed whereas (Deng et al., 2009) suggested 25% to be the minimum cut off for exposed residues. Ren et al. (2014) re-verified 25% as the reliable threshold. In this study the 25% threshold was used to define a residue as being exposed.

2.4. Phylogenetic trees

CLC Genomic workbench v7.5.1 (CLC) was used to construct the phylogenetic trees. This was achieved by importing the filtered sequences and creating a multiple sequence alignment using the very accurate (slow) option. The Create Tree protocol was selected and the aligned sequences were inserted. Neighbor Joining and Kimura Protein methods were used for tree construction and protein distance measurements. For the potential epitope-specific trees, sequences that did not contain the peptides of interest were removed from the aligned sequences. The remaining sequences were then submitted for the construction of the radial phylogenetic trees.

3. Results

To explore the potential of cross-reactive mAbs that target the G protein of *Lyssaviruses*, we have analyzed all G protein sequences of the *Lyssavirus* genus that are available in UniProtKB (UniProt Consortium, 2015). This resulted in the retrieval of 1218 complete G protein sequences from 15 *Lyssavirus* species of which 1139 are from the rabies virus. Table 1 (Ceballos et al., 2013) shows the number of sequences retrieved for each species. We subsequently determined the consensus sequence of the rabies virus G protein sequences and of all the *Lyssavirus* G protein sequences. For the latter, to avoid bias towards the RABV G protein due to their large number, we used the consensus sequence of the RABV G protein sequences during the calculation of the consensus sequence of the *Lyssavirus* genus G protein. Next, Scop3D (Vermeire et al., 2015) was used to calculate the sequence conservation and entropy of all RABV G protein sequences and all *Lyssavirus* G protein sequences. For the latter, we again used the consensus sequence of the rabies virus to avoid bias towards this virus. Fig. 1 gives the sequence

Table 1
Number of Lyssavirus G protein sequences. Number of G protein sequences for each species of the *Lyssavirus* genus that are taken into account during the analysis. The taxonomy is based on the work of Miia and co-workers (Ceballos et al., 2013).

Order: *Mononegavirales*
Family: *Rhabdoviridae*
Genus: *Lyssavirus*

Species	Abbreviation	Number of sequences
Aravan virus	ARAV	1
Australian bat lyssavirus	ABLV	15
Rabies virus	RABV	1139
Bokeloh bat lyssavirus	BBLV	3
Duvenhage virus	DUVV	7
European bat lyssavirus 1	EBL-1	7
European bat lyssavirus 2	EBL-2	5
Ikoma lyssavirus	IKOV	1
Irkut virus	IRKV	2
Khujand virus	KHUV	1
Lagos bat virus	LBV	17
Mokola virus	MOKV	17
Shimoni bat virus	SHIBV	1
West Caucasian bat virus	WCBV	1
Ozernoe lyssavirus (unclassified lyssavirus)	OLV	1

Fig. 1. *Sequence conservation and entropy of the rabies virus and Lyssavirus G protein sequences*. The consensus sequences of the RABV (top sequence) and Lyssavirus G proteins (bottom sequence) were aligned to determine the level of conservation of the characterized epitopes within the RABV variants and *Lyssaviruses*. Sequence conservation is colored with a gradient that ranges from blue, high conservation, to red, low conservation. The color gradient for entropy goes from yellow, high entropy, to green, low entropy. The relative solvent accessibility of both the monomeric and trimeric form of the G protein is colored in a gradient that ranges from light gray, solvent inaccessible, to dark gray, highly solvent accessible. The known antigenic sites are delineated and possible cross-neutralizing epitopes are colored in red.

conservation and entropy at the sequence level mapped on the calculated consensus sequences.

The RABV G protein contains several well-characterized B-cell epitopes: antigenic site I (KLCGVL), the discontinuous antigenic site II (KRA and GCTNLSGFS), antigenic site III (KSVRTWNEI) and G5 (HDFH) (Kuzmina et al., 2013). To map these antigenic sites on the structure of the RABV G protein (Fig. 2), we identified the structure of the vesicular stomatitis virus (VSV) G protein in the pre-fusion conformation (PDB-entry 5i2s (Roche et al., 2006) *via* a BLAST search (Altschul et al., 1990) with the consensus sequence of the RABV G protein against the PDB (Berman et al., 2000) as the closest homologous structure (22.3% sequence identity and 33.6% sequence similarity).

Because of the relatively low sequence identity between the consensus sequence of the RABV G protein and the VSV G protein, we analyzed the conservation of the disulfide bonds. Disulfide

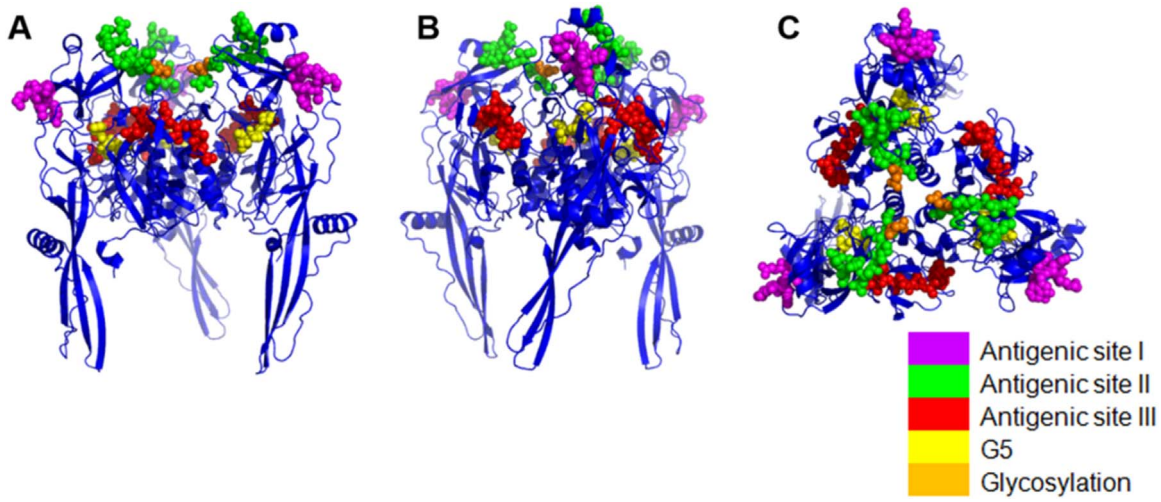

Fig. 2. *Representation of known antigenic sites of the rabies G protein*. The structure of the VSV G protein was used as representative of the rabies G protein (pdb-entry 5i2s (Roche et al., 2006)). A. Cartoon representation of the pre-fusion conformation of the G protein. Each known antigenic site is highlighted with spheres and colored differently. B. Same as A but rotation of 45° along the y-axis. C. View of the top of the G protein.

bonds play a key role in the stability of proteins and are often conserved between homologous proteins (Thangudu et al., 2008). The VSV G protein contains six disulfide bonds: 24-284, 58-92, 68-114, 153-158, 177-224 and 219-253 (numbering according to PDB-entry 5i2s) of which only the disulfide bond between residues Cys 68 and Cys 114 is not conserved within the RABV G proteins as well as within the G proteins of the *Lyssavirus* genus.

As the name implies, the G protein contains several glycosylation sites which often play an important role in antigenicity (Wright et al., 1989). More specifically, the RABV G protein (based on UniProtKB accession P03524) contains three glycosylation sites at positions 56, 266 and 338 of which the latter two are found to be glycosylated in the virion (Shakin-Eshleman et al., 1992).

Scop3D (Vermeire et al., 2015) was used to calculate the

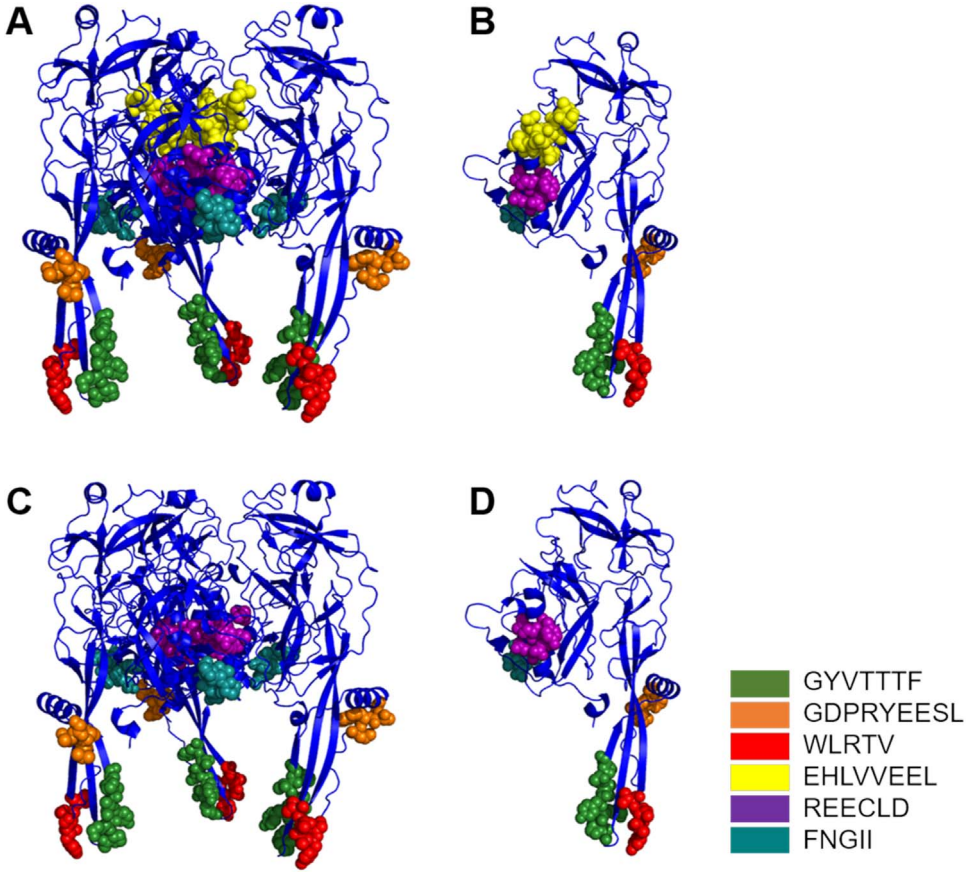

Fig. 3. *Possible cross-neutralizing epitopes of the G protein based on sequence conservation*. The structure of the VSV G protein was used to map the possible cross-neutralizing epitopes (pdb-entry 5i2s (Roche et al., 2006)). The possible cross-neutralizing epitopes are highlighted with spheres and colored differently. A. The G protein in the trimeric pre-fusion conformation with the epitopes for the rabies virus G protein. B. Same as A but the G protein is in the monomeric pre-fusion conformation. C. The trimeric pre-fusion conformation of the G protein with the epitopes for all *Lyssaviruses*. D. Same as C but the G protein is in the monomeric pre-fusion conformation.

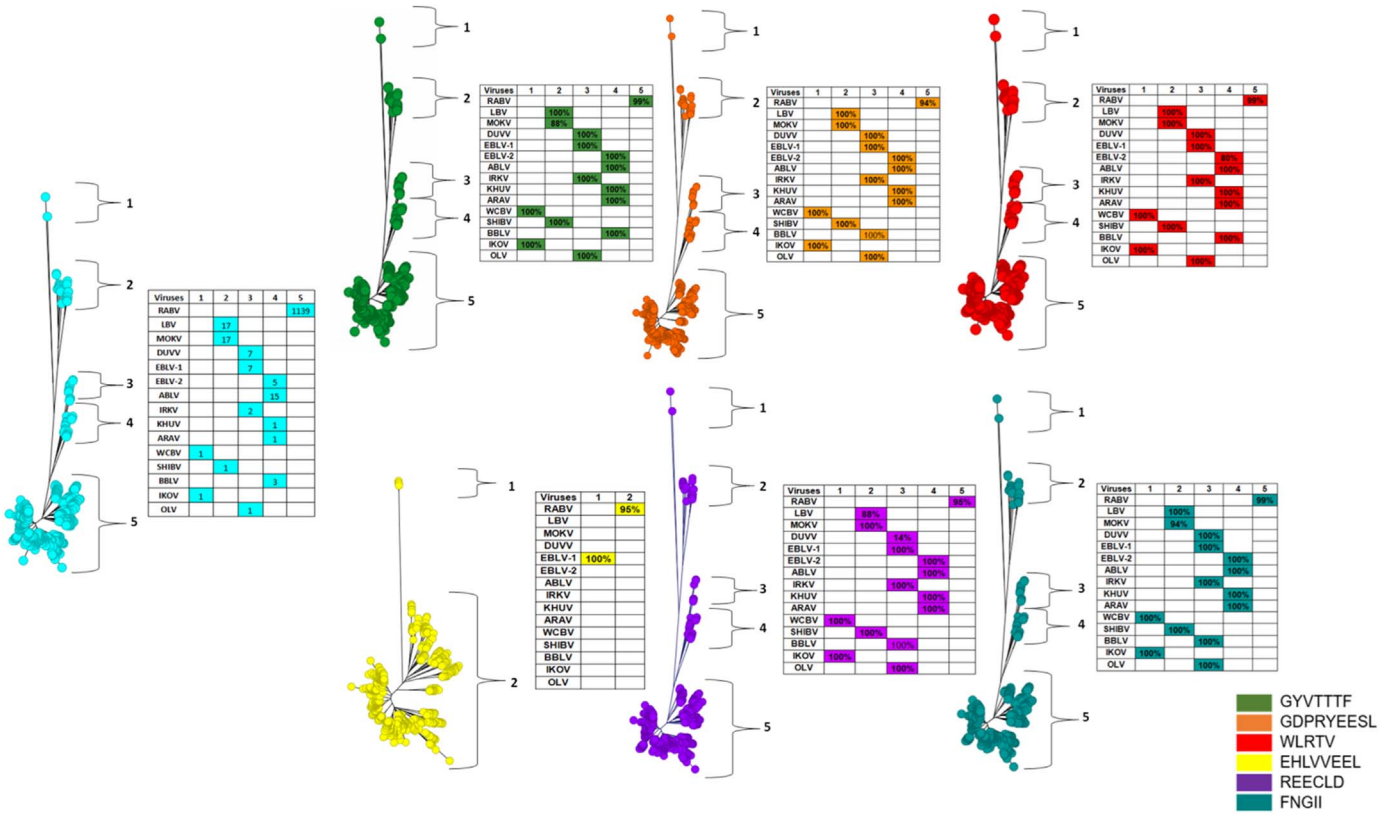

Fig. 4. *Radial phylogenetic trees depicting the percentage of Lyssaviruses that have the indicated conserved regions.* The main radial phylogenetic tree (color: turquoise) of all *Lyssaviruses* used in the analysis is shown in the far left. Next to this main tree are the radial phylogenetic trees (tree colors: green, orange red, yellow, purple and teal) of each of the possible cross-neutralizing epitopes. The tables next to the trees show which viruses possess the conserved region. The percentage was calculated based on the total number of G proteins as listed in Table 1 which is also shown in the table next to the main tree (color: turquoise) at the far left. The node colors correspond to the peptide color coding that is shown at the bottom-right of the figure.

sequence conservation and entropy of each position of the RABV and *Lyssavirus* G protein sequences. The entropy provides information on the random spread of the observed variation across all twenty amino acids. Hence a low entropy value indicates that only few amino acids are possible while a high entropy means that many different amino acids are found. The structure of the VSV G protein (PDB-entry 5i2s) (Roche et al., 2006) was used to visualize both the sequence conservation and entropy. With this information, we identified all possible cross-neutralizing B-cell epitopes and mapped them on the pre-fusion conformation of the G protein (Fig. 3). Regions that display a high sequence conservation and that are surface exposed were defined as possible cross-neutralizing B-cell epitopes (Mansfield et al., 2004). The solvent accessibility was calculated with the aid of DSSP (Kabsch and Sander, 1983; Touw et al., 2015). This value was then used to calculate the RSA (Supplementary Material 3 and 4) (Chothia, 1976). Phylogenetic trees (Fig. 4) were calculated to demonstrate the potential for cross-neutralization of the identified regions.

4. Discussion

Until recently, treatment against rabies infection consisted of PEP with HRiG ERIG (Müller et al., 2009; Yousaf et al., 2012). Since the availability of several characterized mAbs, the WHO proposed to treat rabies infections with a cocktail that is composed of specific combinations of these anti-rabies mAbs, prepared by recombinant expression. One requirement of the cocktail is that it has to address the natural variation among rabies viruses (Marissen et al., 2005). Some of these mAbs are cross-reactive; they also neutralize other members of the *Lyssavirus* genus (Terryn et al., 2014). Broadening the protection of the current anti-rabies cocktail to other rabies-related viruses is highly important (Jallet et al., 1999), as new *Lyssavirus* species that have similar symptoms to a RABV infection are frequently discovered world-wide (Kuzmin et al., 2003). To explore the cross-neutralization potential of the currently available mAbs we have analyzed the conservation within all available G protein sequences of the *Lyssavirus* genus. The conservation was subsequently mapped on the protein structure of a representative of the G protein to explore this protein for other possible cross-neutralizing epitopes.

We identified the G protein of VSV as the closest homologous structure of the G protein of the members of the *Lyssavirus* genus. Like the *Lyssaviruses*, VSV belongs to the family of *Rhabdoviridae* but falls within the *Vesiculovirus* genus. Moreover, a previous study has shown that the G proteins from VSV and the RABV share the same fold (Albertini et al., 2012). Although the sequence identity between the G protein of VSV and that of the rabies virus is relatively low, most disulfide bridges are conserved which additionally supports our choice of template.

The G protein undergoes large conformational changes during its lifetime. At the surface of the virion, the G protein is in the pre-fusion conformation which allows it to bind to its receptors on the host cell. In this conformation, the G protein of VSV can be found both as monomer and trimer (Yousaf et al., 2012). In the first step of the fusion process, upon interaction with the target cell membrane, the G protein adopts the activated hydrophobic conformation (Gaudin et al., 1993; Durrer et al., 1995). Once the G protein is in the acidic environment of the endosome, it adopts the post-fusion conformation and membrane fusion can occur (Gaudin et al., 1993). Due to its uptake pathway, only the pre-fusion conformation of the G protein is visible to antibodies of the host as

this is the conformation which is found in the extracellular environment (Gaudin et al., 1993). We therefore only focused on the pre-fusion conformation to identify possible cross-neutralizing epitopes.

To determine which regions are highly conserved within the G protein of the RABV and broader, within the *Lyssavirus* genus, we calculated the sequence conservation of each position with the aid of Scop3D (Vermeire et al., 2015). Within the RABV, the sequence is overall well conserved apart from the signal peptide, the intramembrane domain and the region that is located within the virion (Fig. 1). These are three regions which are not visible for antibodies and do not have a known important function during the fusion process. The residues that form the intramembrane domain do however have to fulfill the requirement of being hydrophobic.

Analysis of the sequence conservation of the known antigenic sites (Fig. 1) shows that within the rabies viruses these antigenic sites are overall well conserved. Antigenic site I is highly conserved within the RABV. This antigenic site is however much less conserved within the *Lyssavirus* genus. Here only the Cys and Gly are well conserved. Conservation of the Cys is most likely related to the observation that this residue is involved in a disulfide bridge. Similarly to antigenic site I, the discontinuous antigenic site II is also well conserved only amongst the RABV but not within the whole *Lyssavirus* genus. The few residues which are less well conserved have side chains that point towards the protein interior and hence are not visible for antibodies. An important feature of antigenic site II is the presence of a glycosylation recognition pattern (Asn–X–Ser/Thr) within the rabies virus sequences. This pattern is not present in the other *Lyssavirus* members. However, Shakin-Eshleman et al. (1992) demonstrated that this glycosylation site is usually unglycosylated. So it is most likely that the known mAbs bind the unglycosylated antigenic site II and that glycosylation of this site would prevent antibody binding. Antigenic sites III as well as G5 display much more variation, especially within the *Lyssavirus* genus but also within the rabies virus with only one position highly conserved within antigenic site III. In addition, based on structure (Fig. 2), antigenic site G5 is less accessible to antibodies.

Work done by Bakker et al. (2005) demonstrated that the highly potent CR57 mAb which binds to antigenic site I, can be combined with mAb CR4098 which binds to antigenic site III. This indicates that these mAbs do not have binding sites that compete with each other. This is confirmed with the mapping of the antigenic sites on the structure (Fig. 2). Antigenic sites I and III are not close in structure, hence both mAbs can bind the same G protein. The same conclusion can be made for antigenic sites I and II, based on their position within the structure, antibodies can bind both antigenic sites without competition. This validates the finding that a cocktail of the humanized antibodies E559 which binds to antigenic site II and 62-71-3 which binds to antigenic site I increases neutralization of more rabies strains compared to the individual antibodies (Tsekoa et al., 2016) Antibodies that bind to antigenic sites II or III are found to go into competition with each other (Bakker et al., 2005; Kankanamge et al., 2016). Therefore, without taken structural information into account, it was suggested that these antigenic sites are most likely in close proximity to each other. Analysis of the position of antigenic sites II and III on the structure however, reveals that these antigenic sites are not that close together. So what we see on the structure is in disagreement with what is currently described in literature.

In a next step, we analyzed the conservation within the available sequences to determine possible cross-neutralizing epitopes. Regions that display a high conservation in sequence and are solvent accessible were identified as possible epitopes (Fig. 1) (Mansfield et al., 2004). Seven regions were identified as possible cross-neutralizing epitopes. FNGII fulfills both requirements of a cross-neutralizing epitope: it is solvent exposed and highly conserved both for within the rabies species and within the *Lyssavirus* genus. GDPRYEESL is also a good example of a possible cross-neutralizing epitope for the *Lyssavirus* genus although this is less obvious from the structure because the sequences from the *Lyssavirus* genus have an insertion at this epitope compared to the template structure of the VSV G protein. However, this insertion will most likely be solvent accessible. Based on sequence conservation, IPEMQS is another possible cross-neutralizing epitope but based on the available template, this is less obvious due to an insertion within the template. Both GUVTTTF and WLRTV fulfill the requirements of a B-cell epitope but both these possibilities are located very close to the virion which might impede antibody binding. However, several cross-neutralizing mAbs against the haemagglutinin of influenza are known to bind the stem region of haemagglutinin and hence also bind very close to the virion (Throsby et al., 2016). It has been described that the G protein can also be found as a monomer in the pre-fusion conformation (Albertini et al., 2012). This makes the two possible B-cell epitopes REECLD and EHLVVEEL to be cross-neutralizing epitopes as they are also located within the trimer interface but are fully solvent exposed in the monomeric pre-fusion conformation. Binding of an antibody to the monomeric form would hence prevent formation of the trimer and the concomitant initiation of fusion.

5. Conclusion

We have analyzed the amino acid conservation amongst the available sequences of the RABV G protein and more broadly, the sequences of G proteins of the *Lyssavirus* genus. This allowed us to propose several possible cross-neutralizing B-cell epitopes in complement to the already well-characterized antigenic sites. The work reported could facilitate the development of novel cross-reactive mAbs against RABV and even more broad, against possibly all *Lyssavirus* members.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interest

Authors declare that they have no competing interest.

Funding

H.W.D is a South African Research Chair funded by the Department of Science and Technology and the National Research Foundation (Grant 64788).

S.G.B is a Ph.D. student who is funded by the Department of Science and Technology-National Research Foundation: Professional Development Program (DST-NRF: PDP), Council for Scientific and Industrial Research (CSIR) (Grant 78993) and also received the Wood-Whelan Research Fellowship.

Author contributions

S.G.B and E.V. performed the literature search, conceptualized, conducted the experiments and wrote the manuscript. T.L.T., S.H.S., H.W.D., E.C, R.C. and L. M. were involved in the conceptualization, assisted with the writing and revision of the manuscript.

Acknowledgments

E.V. is a postdoctoral research fellow of the Research Foundation Flanders (FWO).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.08.034.

References

Albertini, A.A.V., Baquero, E., Ferlin, A., Gaudin, Y., 2012. Molecular and cellular aspects of rhabdovirus entry. Viruses 4, 117–139.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool, J. Mol. Biol. [Internet]. 1990 [cited 2016 May 16], 215, pp. 403–10. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/2231712⟩.

Bakker, A.B.H., Marissen, W.E., Kramer, R.A., Rice, A.B., Weldon, W.C., Niezgoda, M., et al., 2005. Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual in vitro escape mutants. J. Virol. [Internet]. 2005 [cited 2016 May 16], 79, pp. 9062–8. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/15994800⟩.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., et al., 2000. The Protein Data Bank, Nucleic Acids Res. [Internet]. 2000 [cited 2016 May 16], 28, pp. 235–42. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/10592235⟩.

Ceballos, N.A., Morón, S.V., Berciano, J.M., Nicolás, O., López, C.A., Juste, J., et al., 2013. Novel lyssavirus in bat, Spain. Emerg. Infect. Dis. 19, 793–795.

Champion, J.M., Kean, R.B., Rupprecht, C.E., Notkins, A.L., Koprowski, H., Dietzschold, B., et al., 2000. The development of monoclonal human rabies virus-neutralizing antibodies as a substitute for pooled human immune globulin in the prophylactic treatment of rabies virus exposure. J. Immunol. Methods [Internet]. 2000 [cited 2016 May 16], 235, pp. 81–90. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/10675760⟩.

Chothia C., 1976. The nature of the accessible and buried surfaces in proteins, J. Mol. Biol. [Internet]. 1976 [cited 2016 Apr 1], 105, pp. 1–14. Available from: ⟨http://www.sciencedirect.com/science/article/pii/0022283676901911⟩.

CLC Genomics Workbench 7.5.1 [Internet]. Available from: ⟨https://www.qiagenbioinformatics.com/⟩.

Deng, L., Guan, J., Dong, Q., Zhou, S., 2009. Prediction of protein-protein interaction sites using an ensemble method. BMC Bioinform. 10, 1471–2105.

Durrer, P., Gaudin, Y., Ruigrok, R.W., Graf, R., Brunner, J., 1995. Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses, J. Biol. Chem. [Internet]. 1995 [cited 2016 May 16], 270, pp. 17575–81. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/7615563⟩.

Gaudin, Y., Ruigrok, R.W., Knossow, M., Flamand, A., 1993. Low-pH conformational changes of rabies virus glycoproteins and their role in membrane fusion, J. Virol. [Internet]. 1993 [cited 2016 May 16], 67, pp. 1365–72. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/8437221⟩.

Jallet C., Jacob Y., Bahloul C., Drings A., Desmezieres E., Tordo N., et al., 1999. Chimeric lyssavirus glycoproteins with increased immunological potential, J. Virol. [Internet]. 1999 [cited 2016 Apr 1], 73, pp. 225–33. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/9847325⟩.

John, T.J., 1997. An ethical dilemma in rabies immunisation. Vaccine 15, 12–15.

Kabsch, W., Sander, C., 1983. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers [Internet]. 1983 [cited 2016 May 15], 22, pp. 2577–637. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/6667333⟩.

Kankanamge, P.J., Irie, T., Mannen, K., Tochikura, T.S., Kawai, A., 2003. Mapping of the low pH-sensitive conformational epitope of rabies virus glycoprotein recognized by a monoclonal antibody #1–30-44. Microbiol. Immunol. [Internet]. 2003 [cited 2016 May 16], 47, pp. 507–19. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/12953844⟩.

Kuzmin, I.V., Orciari, L.A., Arai, Y.T., Smith, J.S., Hanlon, C.A., Kameoka, Y., et al., 2003. Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationships according to N, P and G gene sequences. Virus Res. 97, 65–79.

Kuzmina, N.A., Kuzmin, I.V., Ellison, J.A., Rupprecht, C.E., 2013. Conservation of binding epitopes for Monoclonal Antibodies on the rabies virus glycoprotein. J. Antivir. Antiretrovir. 5, 037–043.

Mansfield, K.L., Johnson, N., Fooks, A.R., 2004. Identification of a conserved linear epitope at the N terminus of the rabies virus glycoprotein. J. Gen. Virol. 85, 3279–3283.

Marissen, W.E., Kramer, R.A., Rice, A., Weldon, W.C., Niezgoda, M., Faber, M., et al., 2005. Novel rabies virus-neutralizing epitope recognized by human monoclonal antibody : fine mapping and escape mutant analysis †. J. Virol. 79, 4672–4678.

Müller, T., Dietzschold, B., Ertl, H., Fooks, A.R., Freuling, C., Fehlner-Gardiner, C., et al., 2009. Development of a mouse monoclonal antibody cocktail for post-exposure rabies prophylaxis in humans. PLoS Negl. Trop. Dis., 3:e542

PyMOL [Internet]. PyMOL Version 1.1r1, Schrödinger LLC. Available from: ⟨www.pymol.org⟩.

Ren, J., Liu, Q., Ellis, J., Li, J., 2014. Tertiary structure-based prediction of conformational B-cell epitopes through B factors. Bioinformatics 30, i264–i273.

Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: the European molecular biology open software suite, Trends Genet. [Internet]. 2000 [cited 2016 May 16];16:276–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10827456.

Roche, S., Bressanelli, S., Rey, F.A., Gaudin, Y., 2006. Crystal structure of the Low-pH Form of the Vesicular Stomatitis Virus Glycoprotein G. Science, 80, 313, pp. 187–91.

Rost, B., Sander, C., 1994. Conservation and prediction of solvent accessibility in protein families, Proteins Struct. Funct. Genet. [Internet]. Wiley Subscription Services, Inc., A Wiley Company, 1994 [cited 2016 May 16], 20, pp. 216–26. Available from: ⟨http://doi.wiley.com/10.1002/prot.340200303⟩.

Rupprecht, C.E., Hanlon, C.A., Hemachudha, T., 2002. Rabies re-examined, Lancet Infect. Dis. [Internet]. [cited 2016 May 16], 2, pp. 327-43. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/12144896⟩.

Samanta, U., Bahadur, R.P., Chakrabarti, P., 2002. Quantifying the accessible surface area of protein residues in their local environment, Protein Eng. [Internet]. 2002 [cited 2016 May 16], 15, pp. 659–67. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/12364580⟩.

Shakin-Eshleman, S.H., Remaley, A.T., Eshleman, J.R., Wunner, W.H., Spitalnik, S.L., 1992. N-linked glycosylation of rabies virus glycoprotein. Individual sequons differ in their glycosylation efficiencies and influence on cell surface expression, J. Biol. Chem. [Internet]. 1992 [cited 2016 May 16], 267, pp. 10690–8. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/1587845⟩.

Terryn, S., Francart, A., Lamoral, S., Hultberg, A., Rommelaere, H., Wittelsberger, A., et al., 2014. Protective effect of different anti-rabies virus VHH constructs against rabies disease in mice. Schnell M.J., editor. PLoS One [Internet]. 2014 [cited 2016 Apr 1], 9, pp. e109367. Available from: http://dx.plos.org/10.1371/journal.pone.0109367.

Thangudu, R.R., Manoharan, M., Srinivasan, N., Cadet, F., Sowdhamini, R., Offmann, B., 2008. Analysis on conservation of disulphide bonds and their structural features in homologous protein domain families. BMC Struct. Biol. [Internet]. 2008 [cited 2016 May 16], 8, pp. 55. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/19111067⟩.

Throsby, M., van den Brink, E., Jongeneelen, M., Poon, L.L.M., Alard, P., Cornelissen, L., et al., 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells, PLoS One [Internet]. 2008 [cited 2016 May 17], 3, pp. e3942. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/19079604⟩.

Touw, W.G., Baakman, C., Black, J., Te Beek, T.A.H., Krieger, E., Joosten, R.P., et al., 2015. A series of PDB-related databanks for everyday needs. Nucleic Acids Res. 43, D364–D368.

Tsepo L., Tsekoa, Therese Lotter-Stark, Sindisiwe Buthelezi, Ereck Chakauya, Stoyan H. Stoychev, Claude Sabeta, Wonderful Shumba, Baby Phahladira, Steve Hume, Josh Morton, Charles Rupprecht, Herta Steinkellner, Michael Pauly, Larry Zeitlin, Kevin Whaley and RC, 2016. Efficient in vitro and in vivo activity of glyco-engineered plant-produced rabies monoclonal antibodies E559 and 62-71-3. PloS one, 10.1371/journal.pone.0159313.

UniProt Consortium, 2015. UniProt: a hub for protein information. Nucleic Acids Res. [Internet]. 2015 [cited 2016 May 15], 43, pp. D204–12. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/25348405⟩.

Velasco-Villa, A., Reeder, S.A., Orciari, L.A., Yager, P.A., Franka, R., Blanton, J.D., et al., 2008. Enzootic rabies elimination from dogs and reemergence in wild terrestrial carnivores, United States. Emerg. Infect. Dis. 14, 1849–1854.

Vermeire, T., Vermaere, S., Schepens, B., Saelens, X., Van Gucht, S., Martens, L., et al., 2015. Scop3D: three-dimensional visualization of sequence conservation. Proteomics [Internet]. 2015 [cited 2016 May 15], 15, pp. 1448–52. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/25641949⟩.

WHO, 1997. Recommendations on Rabies Post-Exposure Treatment and the Correct Technique of Intradermal immunization against Rabies 1997.

WHO, 2005. WHO Technical Report Series 931 WHO EXPERT CONSULTATION ON RABIES First Report 2 WHO Library Cataloguing-in-Publication Data.

WHO, 2013. WHO Expert Consultation on Rabies.

Wright, K.E., Salvato, M.S., Buchmeier, M.J., 1989. Neutralizing epitopes of lymphocytic choriomeningitis virus are conformational and require both glycosylation and disulfide bonds for expression, Virology [Internet]. 1989 [cited 2016 May 16], 171, pp. 417–26. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/2474891⟩.

Yousaf, M.Z., Qasim, M., Zia, S., Khan, M. ur R., Ashfaq U.A., Khan S., 2012. Rabies molecular virology, diagnosis, prevention and treatment, Virol. J. [Internet]. BioMed Central Ltd, [cited 2016 May 15], 9, 50. Available from: ⟨http://www.ncbi.nlm.nih.gov/pubmed/22348291⟩.