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REVIEW

Rabies vaccine development by expression of recombinant viral glycoprotein

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Abstract The rabies virus envelope glycoprotein (RVGP) is the main antigen of rabies virus and is the only viral component present in all new rabies vaccines being proposed. Many approaches have been taken since DNA recombinant technology became available to express an immunogenic recombinant rabies virus glycoprotein (rRVGP). These attempts are reviewed here, and the relevant results are discussed with respect to the general characteristics of the rRVGP, the expression system used, the expression levels achieved, the similarity of the rRVGP to the native glycoprotein, and the immunogenicity of the vaccine preparation. The most recent studies of rabies vaccine development have concentrated on *in vivo* expression of rRVGP by viral vector transduction, serving as the biotechnological basis for a new generation of rabies vaccines.

The rabies vaccine and the rabies virus glycoprotein (RVGP)

Rabies is one of the most fatal diseases caused by viral infection in humans. With few exceptions, humans that develop symptoms of rabies virus infection inevitably die. Like other members of the genus *Lyssavirus*, family *Rhabdoviridae*, rabies virus is a negative sense, single-strand RNA virus carrying five proteins: a nucleoprotein, a phosphoprotein, a matrix protein, an envelope glycoprotein (RVGP) and a viral polymerase [1] (Fig. 1). The structure of the glycoprotein of vesicular stomatitis virus, a well-studied member of the family *Rhabdoviridae*, has recently been determined [2, 3]. Since then, considerable insight has been gained into rhabdovirus structure [4] and virus entry mechanisms [5]. Nevertheless, due to essential differences in the immune mechanisms involved in infections by different rhabdoviruses, studies related to rabies vaccine development need to involve the RVGP directly.

Classical rabies vaccines consist of whole inactivated viruses, that have the same antigenic characteristics as wild type viruses. Immunization with whole inactivated virus has been shown to induce virus-neutralizing antibodies directed against RVGP, activation of helper and cytotoxic T cells and protection against lethal intracerebral challenge with rabies virus [6, 7]. The main reason that further research toward a new rabies vaccine candidate is needed is the high cost of producing rabies vaccine in rabies-virus-infected cell culture [8, 9]. In some developing countries with high incidence of rabies, it is necessary to have a less expensive vaccine, allowing preventive immunization, preferentially after a single dose [10, 11]. Other important reasons include the risks of production and administration of the current whole inactivated virus vaccine and the logistic concerns of a multi-vaccination schedule for pre- and, particularly, post-exposure vaccination [12, 13].

The RVGP is the only antigen able to confer full protection against rabies [14] and is the only component present in all new rabies vaccines that have been proposed [10]. When properly folded and glycosylated [15, 16], the RVGP molecule (Fig. 1B and C) is fully immunogenic, bearing epitopes for humoral and cell-mediated immune responses [6, 7, 17–19]. It has been shown that RVGP is an important determinant for the induction of innate immune responses and different pathogenic mechanisms induced by different rabies virus strains [20].

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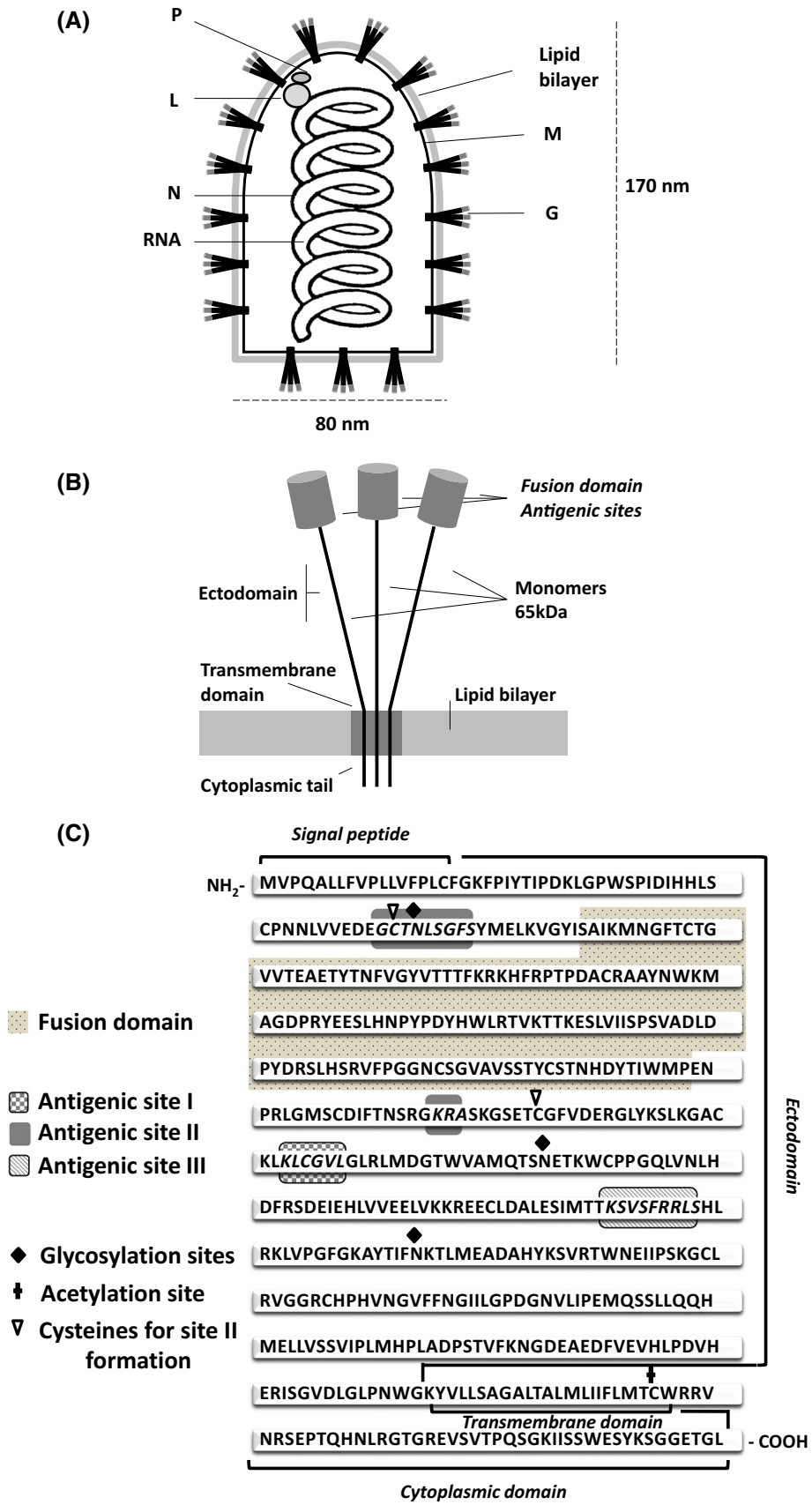

Fig. 1 Schematic representation of rabies virus virion (A), glycoprotein structure (B), and amino acid sequence (C). P, phosphoprotein; L, large protein/polymerase; N, ribonucleoprotein; RNA, non-segmented, negative-strand genomic RNA; M, matrix protein; G, glycoprotein

The native RVGP is located in the rabies virus envelope and the plasma membrane of infected cells before virus budding. The native RVGP is a 505-amino-acid, 65-kDa glycoprotein that contains an intracytoplasmic region, a hydrophobic transmembrane region and an ectodomain region (Fig. 1B and C). The association of three RVGP monomers results in the homotrimerization of the molecule [21] (Fig. 1B and C). The oligomerization state of RVGP seems to be essential for interaction of rabies virus with target membrane receptors [22, 23] and, to a certain extent, for the induction of neutralizing antibodies leading to protection against rabies virus infection [24–27]. The RVGP is a special molecule that combines characteristics of class I and II virus fusion proteins [28]. It is able to bind to at least three different receptors, allowing virus endocytosis [29, 30]: the neurotrophin receptor (p75NTR) [31, 32], the nicotinic acetylcholine receptor [33], and the neural cell adhesion molecule [34]. Virus-cell membrane fusion mediated by RVGP through hydrophobic interactions occurring in a low-pH environment completes the infection process after virus penetration [35, 36]. It has been shown that the RVGP can be found in three different antigenic conformations: a 'native' state, an activated hydrophobic state, and a fusion-inactive state [22, 36]. In addition to the fact that these different RVGP conformations are very important for the processes of virus budding and fusion, they present different epitopes and are not equally recognized by neutralizing antibodies directed against mature or native RVGP [36].

Beyond its utilization for virus infection studies and anti-rabies vaccination, RVGP has also been used for studies of the nervous system. It has been used for mapping or tracing neuronal connections for better understanding nervous system processes [37]. Recently, RVGP-derived peptides have been utilized to deliver siRNA to specific neuronal cells or macrophages expressing acetylcholine or GABA receptors [38–41] or to deliver therapeutic proteins to the central nervous system [42–44]. In the field of toxinology, the affinity of snake neurotoxins for the acetylcholine receptor has been studied in comparison with an RVGP-derived peptide [45] as well as the whole RVGP as an antagonist [45].

The potential use of a stable, oligomerized form of an rRVGP for virus infection or nervous-system studies and immunization purposes has justified new research approaches for the establishment of recombinant systems for expression of rRVGP [9].

rRVGP expression in cell systems

The pathway to the establishment of an expression system able to produce promising levels of a high-quality rRVGP also includes the determination of how close the rRVGP is to a specific native RVGP. Despite the availability of modern biochemical analytical tools, the best method of comparison is still the analysis of the immune response upon animal inoculation. It is well known that the immunogenic quality of the recombinant molecule itself is dependent on its oligomeric structure [46, 47]. Many approaches for producing high levels of the immunogenic form of rRVGP have the problem that one is working with an unstable and hydrophobic molecule. After synthesis, RVGP undergoes post-translational modifications, and at least one sequon must be glycosylated to allow the RVGP to reach the cell surface [48]. As rRVGP is not primarily responsible for virus budding [49], it does not protrude in the form of vesicles, remaining in plasma membrane until cell lysis [49]. In early studies, the discovery of the importance of the plasma membrane and, consequently, the viral envelope for RVGP stability led to research on new vaccines based on a RVGP presented in liposomes [14]. To avoid the difficulties caused by hydrophobicity, some genetic and biochemical approaches were tried in order to obtain a vaccine constituted from an immunogenic soluble form of rRVGP, or just the soluble ectodomain. However, despite the potential value of soluble forms of rRVGP as diagnosis tools [50, 51], these approaches produced only poorly immunogenic molecules [46, 52, 53] and suggested an essential role of the transmembrane domain in the correct folding of the ectodomain, where the most important epitopes are located [47] (Fig. 1). Further studies using a DNA vaccination approach showed that the native transmembrane domain was required for an adequate humoral immune response [54]. Additionally, immunization with RVGP-derived peptides bearing predicted or mapped epitopes generated antibodies of only moderate immunogenicity [55]. The complete failure of these attempts to produce a soluble and immunogenic form of rRVGP or an rRVGP with a more stable transmembrane domain stressed the importance of research and development projects to establish an expression system capable of producing high levels of functional native-like rRVGP [56–59].

Given the wide distribution of rhabdoviruses in nature and the act that membes of this viral genus are capable of infecting plants, mammals, and also insects, it is reasonable to investigate rRVGP expression using different systems. In fact, rRVGP has been expressed in many cell systems, showing encouraging results in immunization studies [60–62]. Eukaryotic systems are appropriate for rRVGP expression, as it is known that only the N-glycosylated rRVGP is transported from Golgi complex to the cell membrane [63] and that rRVGP folding and glycosylation patterns are important for its immunogenicity [15, 64]. The RVGP has three sequons for potential N-glycosylation (Asn-X-Ser/Thr) in the ectodomain: Asn37, Asn247, Asn319 [65]. In general, expression in eukaryotic systems,

by virus infection or recombinant means, produces an rRVGP with efficiently glycosylated Asn247 and Asn319 [48]. The glycan composition is dependent on the biochemical machinery of the host cell and seems to be a determinant of immunologic properties. For example, when rRVGP was expressed in *Saccharomyces cerevisiae,* the rRVGP was found to be associated with the yeast membrane and was able to protect guinea pigs but not mice against lethal challenge [66]. The authors argued that the difference was a consequence of immunizing animals by different routes. More likely, the characteristic high-mannose glycosylation pattern of yeast [67] was not appropriate for rRVGP stabilization and full immunogenic properties. Further studies also showed that this rRVGP was not processed normally, resulting in abnormal folding and multimer formation. These observations strongly discouraged new approaches for producing full-length rRVGP in yeast systems [68]. More recently, it was demonstrated that a trimeric rRVGP ectodomain produced in *Hansenula polymorpha* had good antigenic properties and was suitable for use as an antigen in diagnostic tools [51].

The rRVGP has also been expressed in plants [69–75]. This approach in general results in properly folded and glycosylated rRVGP, as is the case in *Agrobacterium tumefaciens*-transformed tomato plants expressing the rRVGP under the control of a cauliflower mosaic virus promoter [76]. The rRVGP was found in leaves and fruit by immunoprecipitation and western blotting methods [77]. High-level rRVGP expression in tobacco plants was achieved by using genetic engineering techniques, leading to rRVGP being retained in the endoplasmic region [77]. Surprisingly, a glycoprotein form that was not attached to the cytoplasmic membrane showed a high level of immunogenicity in intraperitoneally immunized mice when compared to the commercial vaccine [77]. It is claimed that the main advantage of producing rRVGP in plants is the possibility of oral delivery. For this purpose, rRVGP was combined in a chimeric peptide containing antigenic determinants from RVGP and rabies virus nucleoprotein and cloned as a translational fusion product with the alpha mosaic virus (AlMV) coat protein (CP) [12]. Spinach (*Spinacia oleracea*) plants infected with AlMV recombinants were then successfully used for oral-boosting anti-rabies vaccination, protecting mice against challenge infection [12]. However, in the context of a post-exposure vaccination, when a rapid and intense immune response is needed, the oral immunization may not be adequate for rabies prevention, restricting oral vaccination with rRVGP to pre-exposure immunization [12].

Promising insect-cell-based systems have also been evaluated for rRVGP expression [56, 78, 79]. The expression of rRVGP in *Spodoptera frugiperda* (Sf9) cells using a recombinant baculovirus with the rRVGP gene under the control of a polyhedrin promoter produced a glycoprotein with good structural and immunogenic characteristics when administered anchored to the cytoplasmic membranes of baculovirus-infected cells [78]. The potential use of rRVGP produced in a baculovirus–insect cell system was further evaluated more recently, when a purified form of rRVGP produced in Sf9 cells infected with a recombinant baculovirus was found to be immunogenic when tested in mice, as evidenced by high virus-neutralizing antibody titers in sera and 100% protection upon virulent intracerebral challenge [56].

Drosophila melanogaster Schneider 2 (S2) cells have been intensively studied as host cells for rRVGP production [79]. Many aspects related to rRVGP expression in different media, controlled culture conditions with different substrate concentrations, pH, temperature and oxygenation and their consequences for rRVGP productivity have been described [79–83]. The rRVGP produced in S2 cells was oligomerized and immunogenic, protecting mice against challenge infection with rabies virus [58].

The use of mammalian cells in large-scale processes for producing rabies virus for vaccination is the basis of many second-generation rabies vaccines. In this context, it is of note that there are only a few reports of stable rRVGP expression in mammalian cells. One reason is that the glycosylation pattern of rRVGP might also be critical in mammalian systems. As for many other recombinant proteins, it depends on the cell type used and may change with cell culture conditions [84, 85]. For example, when both neuroblastoma cells (NA) and baby hamster kidney cells (BHK-21) were transfected with a vector derived from retroviruses for rRVGP expression, only the glycoprotein expressed in BHK-21 cells was correctly glycosylated. Furthermore, rRVGP expressed constitutively in BHK-21 cells and that produced after rabies virus infection showed different glycosylation patterns [86]. In fact, the quality of the expressed rRVGP should be carefully considered. It was demonstrated by another group that, in BHK-21 cells, the formation of essential rRVGP epitopes was dependent on culture conditions [64]. Similar results were found when comparing rRVGP expression in COS-1, neuroblastoma, and BHK-21 cells, where different glycosylation patterns were found that were due to the influence of host factors [52, 87]. Finally, rRVGP expression in CHO cells, possibly the most-used mammalian cell expression system, has been used preferentially for glycosylation studies rather than for immunization purposes [48, 63, 88, 89].

Reverse genetics, a powerful tool for studying functions of genes, has been increasingly used for molecular engineering of RNA viruses. The potential for applying this approach to rabies virus dates from the introduction of reverse genetics to virus investigation [90, 91]. Since then, great progress has been made in RNA virus reverse

genetics and vaccine design [92]. The possibility of utilizing reverse genetics for attenuation of rabies virus and construction of viral vectors can be envisaged, and this opens promising perspectives in rabies vaccinology [93].

rRVGP purification

Another important feature of the expression of rRVGP on cell membranes for vaccination purposes is the requirement for an efficient purification process. The purification of native RVGP from virus suspensions is a well-established process [94]. It is based on the ultracentrifugation of cell supernatant after virus budding for isolation and concentration of rabies virus [94]. Following virus dissociation with a detergent-containing buffer, a new ultracentrifugation in a sucrose gradient is performed for the separation of viral proteins [94, 95]. Immunoprecipitation is another technique that has been used to purify RVGP [15]. The drawback associated to this methodology is that, in general, the oligomerization status of the glycoprotein is compromised, so it is a technique of choice mainly for analytical purposes [15, 30, 87]. Although these methodologies are very efficient for native RVGP purification or analysis, they are not optimal for the application of rRVGP to immunization studies in which rRVGP has to retain its immunogenic properties [30, 66, 86, 96]. The challenge in rRVGP purification is to separate the rRVGP from other cell proteins while retaining its trimeric structure and important epitopes [97]. This process is generally conducted in a detergent environment to avoid aggregation and precipitation of rRVGP [95]. Several different detergents have been used to solubilize the rRVGP. The first detergent broadly that was used was Triton X-100 [95], but it was later demonstrated that it caused some denaturation of rRVGP, and the use of CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) allowed the trimeric state of rRVGP to be retained [21]. Other detergents were also tested, but the best results for rRVGP trimer solubilization were achieved with CHAPS or OGP (octyl β-D-glucopyranoside) [56, 94].

The fusion of rRVGP with purification tags is an approach that is only rarely used. The histidine tag–IMAC strategy was applied for the purification of a truncated form of rRVGP without a transmembrane domain [53] and for an rRVGP under denaturing conditions [52]. In both cases, the resulting rRVGP was not immunogenic. Purification methods based on FPLC and ion exchange columns or gel filtration were also used [98] with low recovery of trimeric rRVGP. The best progress on rRVGP purification was achieved by those working with tobacco leaves. When extracted from plant cells and purified by ion exchange chromatography followed by immunoaffinity [77] or concanavalin A affinity chromatography [96], the rRVGP maintained its conformation and immunogenicity in mice. However, it is clear that the current procedures for purification of rRVGP from cell membranes are more laborious than purification of RVGP from the virus. Additionally, expression levels of membrane protein in recombinant animal cell systems are generally low in comparison to those of soluble proteins [99, 100]. The low rRVGP expression levels attained using different cell systems, even when using strong DNA promoters, is another difficulty of this approach [56, 86, 101]. Altogether, these drawbacks usually make expression of rRVGP in cell systems laborious and discouraging for further vaccination studies.

Viral vectors for RVGP expression

Another strategy for development of a new rabies vaccine that has been studied is the use of a viral vector [102–111, 113–122, 124–128]. Many viruses were genetically engineered for rRVGP expression *in vivo*. The most successful program of rabies wildlife immunization is based on an oral vaccine consisting of a mildly attenuated recombinant vaccinia virus (VACV) expressing the RVGP gene [82]. For nearly 20 years, this rRVGP viral vector system has been used to immunize red foxes, raccoons, coyotes and skunks and has been crucial for the elimination of rabies in parts of Europe and the significant reduction of the incidence of rabies in the United States [103, 104]. In addition to VACV, adenovirus-based vectors expressing rRVGP have also been proposed for immunization of wildlife [105, 106]. This recombinant respiratory virus was shown to induce high levels of rabies-virus-neutralizing antibodies, and 100% of immunized mice survived a lethal rabies virus challenge [107]. The development of recombinant adenovirus-based rabies vaccines has led to a number of studies in which the recombinant adenovirus and vaccinia virus systems were compared. For example, the vaccinia vector was successfully utilized for anti-rabies immunization of raccoons (*Procyon lotor*) in a very large program of wildlife vaccination [86], and the immunization of raccoons with a rRVGP adenovirus-based vaccine also resulted in protection in trials [105, 106, 109]. Another recent study showed that an adenovirus-based rabies vaccine may be more effective than the traditional vaccinia-based vaccine for the immunization of raccoons [110]. However, studies have shown that neither vaccine is appropriate for immunization of striped skunks (*Mephitis mephitis*), and therefore the appropriate recombinant vectors to use for anti-rabies immunization is still a matter of debate.

Although genetically modified rabies virus strains have been shown to induce long-lasting protective immune responses in animals [111, 112], researchers have studied

new viral vectors for veterinary rabies immunization [113–115]. The most important animal in the epidemiology of rabies worldwide is undoubtedly the dog. Low doses of recombinant Newcastle disease virus (NDV) expressing rRVGP protected dogs from challenge with a street rabies virus for more than one year, suggesting that immunization with an NDV-vectored vaccine can induce long-lasting, systemic protective immunity against rabies in dogs [113]. Another study showed that canine herpesvirus (CHV), when used as a live vector for the expression of rRVGP after intranasal inoculation in dogs, produced higher titers of neutralizing antibodies against rabies virus than a commercial, inactivated rabies vaccine [114]. For broad veterinary immunization, poxvirus-based rabies vaccines were considered very promising and have been proposed several times, especially with raccoon poxvirus [115]. Nevertheless, the limitations of their efficacy in some target species and poor results of oral vaccination have discouraged further studies with these vectors [116]. Other virus vectors expressing rRVGP that have been proposed mainly for veterinary immunization include vaccinia virus Ankara [117], canine adenovirus [118], canine distemper virus [119], parainfluenza virus 5 (administered by the intranasal route) [96], canary pox virus recombinant (for mucosal priming effect) [121] and baculovirus (for oral or systemic immunization) [30, 78, 122].

It is important to note that the efficacy of immunization against rabies with a viral vector is almost always evaluated based on neutralizing antibody, cytotoxic T cell activation and challenge protection. However, as it occurs *in vitro*, the conditions for rRVGP production *in vivo* may vary, influencing the quality and quantity of rRVGP. The description of the correlations between RVGP levels expressed by a rabies virus strain and its pathogenicity and immunogenicity [87] stimulated some studies with the goal of increasing rRVGP expression by using a pseudorabies viral vector [123], which, in general, increased immunogenicity. Also, the improved rRVGP presentation by recombinant inactivated Flury low-egg-passage rabies virus resulted in higher levels of neutralizing antibodies [124]. A parapoxvirus (ORF) recombinant expressing rRVGP was used for rabies immunization of mice, dogs and cats, inducing high levels of neutralizing antibodies and providing good protection in mice after intracerebral challenge [125]. The amounts of recombinant protein produced *in vivo* after immunization were not estimated, but a direct correlation between the virus dose and neutralizing antibodies suggested that the rRVGP levels were important for the development of a protective immune response [87, 126].

These studies show that when planning a vectored rRVGP vaccine, the amount of *in vivo*-produced and/or delivered rRVGP is an important feature to take into account. In this context, a promising Semliki Forest virus vector carrying an RVGP mRNA (SFV-RVGP) was shown to be capable of inducing very high levels of rRVGP in cell cultures [59]. Upon immunization with SFV-RVGP, mice were shown to develop a strong humoral and cellular immune response [127]. The same principle of delivering an mRNA encoding RVGP using a viral vector was used in the delivery of an mRNA adjuvanted with protamine, which was able to induce potent neutralizing antibodies and protection in mice and domestic pigs [128].

Final remarks

The complexity of the oligomeric rabies virus glycoprotein expressed on cell membranes hampers the studies of its structure and function as well as the establishment of a vaccine based on immunogenic rRVGP produced in cell culture. It is quite well established that the trimeric form of RVGP is necessary for infection through receptor binding and for induction of a protective immune response. There is ample evidence that, for vaccine purposes, the characteristics of the native RVGP have to be maintained during expression and purification of rRVGP and vaccine formulation. On the other hand, in terms of vaccine design, the *in vivo* expression of rRVGP by a virus vectored for gene delivery has been shown to be a more straightforward strategy, since the rRVGP synthesized *in vivo* is more likely to possess the required structure and antigenicity. The available results of studies of the immunogenicity of viral vectors expressing the rRVGP are very encouraging. A rabies vaccine based on rRVGP would contribute to simplifying the industrial bioprocess, quality control, and endemic rabies control.

Compliance with ethical standards

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Conflict of interest The authors (Renato M. Astray, Soraia A. C. Jorge and Carlos A. Pereira) declare that they have no conflict of interest.

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