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Review

Development of new therapeutical/adjuvant molecules by pepscan mapping of autophagy and IFN inducing determinants of rhabdoviral G proteins

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ABSTRACT

Surface glycoproteins of enveloped virus are potent elicitors of both innate and adaptive host immune responses. Therefore, the identification of viral glycoprotein determinants directly implicated in the induction of these responses might be of special interest for designing new therapeutical/adjuvant molecules. In this work we review the contribution of the "pepscan" approach to the screening of viral functions in the sequence of glycoprotein G (gpG) of the fish rhabdovirus of viral hemorrhagic septicemia (VHSV). Among others, by scanning gpG peptides, it has been possible to identify and validate minimal determinants for gpG directly implicated in initiating the fish type I Interferon-associated immune responses as well as in the antiviral autophagy program. Further fine-tunning of the identified peptides in the gpG of VHSV has allowed designing novel adjuvants that decrease DNA vaccine requirements and identify possible innovative antiviral molecules. In addition, these results have also contributed to improve our knowledge on how to stimulate the fish immune system.

1. Introduction

In 2012, the Food and Agriculture Organisation (FAO) published its latest report on the State of the World Fisheries and Aquaculture (FAO, 2012). In this document, FAO reports that in 2011 the aquaculture industry produced more than 60 out of the 154 million tonnes of fish supplied worldwide by capture fisheries and fish farms combined. In fact, the steady growth of the aquaculture industry seen in the last three decades responds to a higher demand for fish products and the inability of capture fisheries to meet this demand (FAO, 2012). Today, cultured fish production is led by Asia (89% of the world's total production) followed by the Americas (4.3%) and Europe (4.2%) (FAO, 2012).

For the aquaculture industry to supply the growing demand for fish and other aquatic species in the future, advances are needed in different fronts. The need to prevent outbreaks of infectious disease in fish farms is one of the most important. In Europe, diseases caused by aquatic RNA viruses, in particular, fish rhabdoviruses, have the largest negative impact on aquaculture (Gomez-Casado et al., 2011). In addition, the problem of aquatic viral diseases is aggravated for two reasons (i) the lack of treatments to cure and resolve viral outbreaks, such as antibiotics in the case of bacterial diseases and (ii) the need for further development of efficacious preventive methods, such as vaccines, in order to meet the requirements imposed by national health and drug agencies.

In the last two decades, DNA vaccines have emerged as an alternative to prevent fish diseases caused by fish rhabdoviruses (Evensen, 2013). Efficacious DNA vaccines against the viral hemorrhagic septicemia (VHS) and infectious hematopoietic necrosis (IHN), which affect salmonid species, have been developed based on the gene sequence of the envelope glycoprotein G of the viruses (gpG) that cause these diseases, the viral hemorrhagic septicemia (VHSV) and the infectious necrosis viruses (IHNV). While these vaccines have become the benchmark of DNA vaccine efficacy (Chico et al., 2009; Evensen, 2013; Gomez-Casado et al., 2011) and one of them, Apex-IHN® (Novartis Aqua Health), has been licensed and commercialized in Canada for its use in Atlantic salmon (Salmo salar) farms (Alonso and Leong, 2013; Salonius et al., 2007) DNA vaccines against other rhabdoviruses have not shown the same

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degree of efficacy. In this context, DNA vaccines against the Spring Viremia of Carp (SVC), caused by the SVC virus (SVCV), have failed to meet the standards set by VHSV and IHNV vaccines (Gomez-Casado et al., 2011). SVCV poses a major threat to cultured carp producers in central and Eastern Europe, and, in recent years, has spread worldwide (Padhi, 2012). Attempts to develop different DNA vaccines encoding the glycoprotein G of SVCV (gpSVCV) have resulted in relatively low protection levels in common carp (Cyprinus carpio) (Kanellos et al., 2006) and ornamental koi (Cyprinus carpio koi) (Emmenegger and Kurath, 2008) (48 and 50–88 relative percent survival [RPS], respectively) using relatively high vaccine doses (25 and 10 μg of DNA per fish, respectively). The reasons for the lack of efficacy of DNA vaccines against SVCV, despite its close phylogenetic relatedness with VHSV and IHNV, are yet to be elucidated. Thus, efforts aimed at identifying differences in the response of fish to these viruses and their antigens (gpGs) will provide useful information to develop new, more efficacious and cost-effective vaccines against SVCV as well as against other fish virus. Likewise, a deeper understanding of host-virus interactions is needed to help the rational design of new vaccines and adjuvants. Furthermore, these results have also contributed to improve our knowledge on how to stimulate the fish immune system.

On the other hand, recent years have witnessed a revitalization in the study of the fish immune system because it have been useful for increasing the information of the evolution of vertebrate immune systems and for providing information for the discovery of previously unknown molecules and biochemical pathways involved in mammalian (Sunyer, 2013).

2. Fish *Rhabdoviruses* and their surface glycoprotein G (gpG)

As stated before, fish *rhabdoviruses* cause the most important viral diseases in worldwide fresh and marine water farming and are being isolated from many other fish species captured from the ocean (Olesen, 1998; Skall et al., 2005).

The *Rhabdovirus* particle (Fig. 1) contains a genome of a single RNA molecule of negative polarity of ~11 kbp (Hill et al., 1975; Schutze et al., 1999), coding for 6 structural proteins (L, N, P, M and gpG). Nevertheless, the fish rhabdovirus affecting salmonids, VHSV and IHNV, together with other piscine rhabdoviruses, have been placed into the *Novirhabdovirus* genus because of the presence of an additional gene encoding for a non-virion (NV) protein. The gene encoding NV (12 kDa) is localized between the gpG and L genes (3′ N-P-M-gpG-NV-L 5′) (Thoulouze et al., 2004). NV is absent in other fish, mammalian or plant rhabdoviruses (Basurco and Benmansour, 1995; Essbauer and Ahne, 2001 Schutze et al., 1996).

Inside the rhabdoviral particle, the RNA genome is tightly packed by the nucleocapsid N protein, associated with the RNA-dependent RNA polymerase (L) and the P proteins, to form the replication complex. The matrix M protein, is localized between the inner leaflet of the surrounding lipid bilayer membrane and the nucleocapsid. The viral membrane is a lipid bilayer derived from the host cell, containing ~400 spikes of trimers of gpG monomers (Coll, 1995). The gene coding for the glycoprotein G (gpG) forms the protruding transmembrane trimeric spikes (Fig. 1) of the virions, one of the main targets of actual biotechnological methods to interfere with rhabdoviral infections in the aquatic media.

Among the VHSV proteins, gpG has probably received most interest due to their role at viral entrance, fusion and because is the only rhabdoviral protein capable of inducing early and late responses such as neutralizing antibodies (NAb), in the fish host during infection (Boudinot et al., 1998; Lorenzen et al., 1990).

The fish *Rhabdoviral* gpGs are ~500 amino acid residues long. Despite the fact that fish and mammalian gpGs share only 18–26%

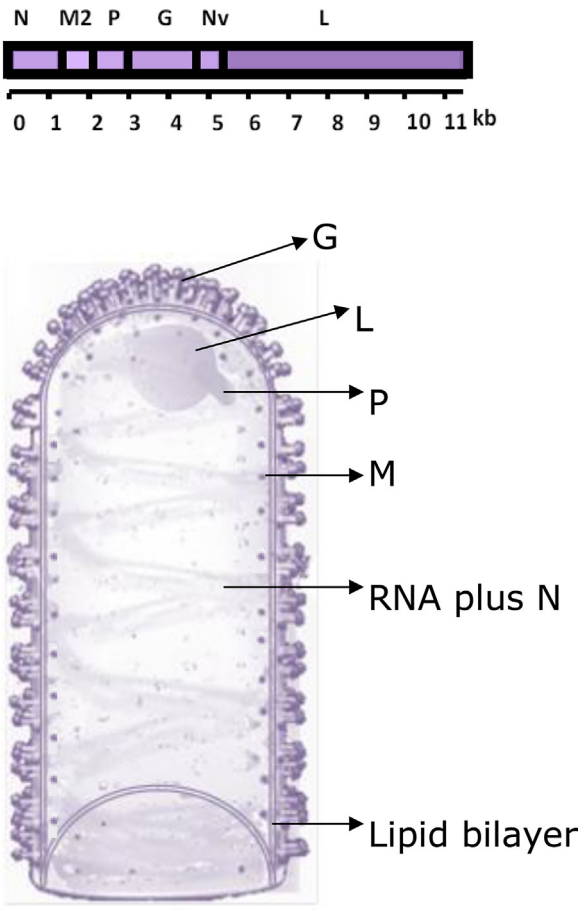

Fig. 1. Schemes of Rhabdoviral particles. The Rhabdoviral particle (170 × 80 nm) is bullet shaped and contains an RNA molecule of negative polarity coding for 5 structural proteins (L, N, P, M and G). A non-virion protein (NV) is present only in the group of *Novirhabdoviruses*. The RNA-dependent RNA polymerase, L (190 kDa) and the nucleocapsid N (40 kDa) and P (19 kDa) form the replication complex sourrrounded by a lipid membrane. About 400 trimeric transmembrane spikes of glycoprotein G (gpG) (65 kDa) protrude from the membrane.

homology, their sequences have similar features (Roche et al., 2006; Walker and Kongsuwan, 1999). Conserved features do include a signal peptide hydrophobic sequence (removed in the mature gpG), 2–3 *N*-glycosilation sites, >10 disulphide bonds, a transmembrane region, 2–3 non-cannonical hydrophobic heptad repeat-like sequences and a carboxy-terminal cytoplasmic tail (Coll, 1995). After endocytosis by the host cell, gpG mediates the fusion between viral and host membranes (Gaudin et al., 1993), following extensive conformational rearrangements triggered by the low pH of the endosomes (Albertini et al., 2012; Gaudin et al., 1999). An alignment model based on the highly conserved cysteines, identified homologous putative fusion domains in other 14 animal rhabdoviruses (Walker and Kongsuwan, 1999). Contrary to many other enveloped viruses, in rhabdoviruses there is no proteolytic processing of gpG to expose the so called fusion domain and the conformational changes required for low pH fusion are reversible (Albertini et al., 2012; Doms et al., 1987; Roche and Gaudin, 2002). The fusion domain contains 2 loops (Albertini et al., 2012) and an internal cysteine (except in rabies virus) (Fredericksen and Whitt, 1996 Shokralla et al., 1998).

In 2006–2007 the crystal structure of the gpG ectodomain (residues 1–410) of the prototypical mammalian rhabdovirus vesicular stomatitis virus (VSV) at physiological and fusion pHs (Roche et al., 2006, 2007) was dilucidated. From that data, the tridimensional (3D) location of structural/functional features of gpG_{VSV} as

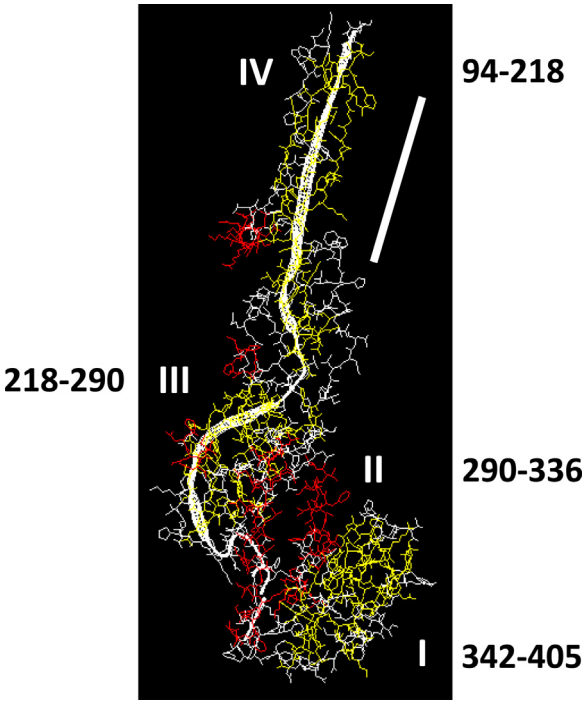

Fig. 2. Localization in the modeled structure of gpG_{VHSV} (numbered with the signal peptide) at the low pH conformation of gpG_{VSV} I–IV domains. The globular head folds into 3 domains (domains I–III) made up of discontinous segments (Roche et al., 2006, 2007), which suffer most of the extensive conformational changes with low pH. The fusion domain (domain IV) is made of a continous segment folded in 2 fusion loops crosslinked by a disulphide bridge (52–180 in gpG_{VSV} corresponding to 94–216 in gpG_{VHSV}).

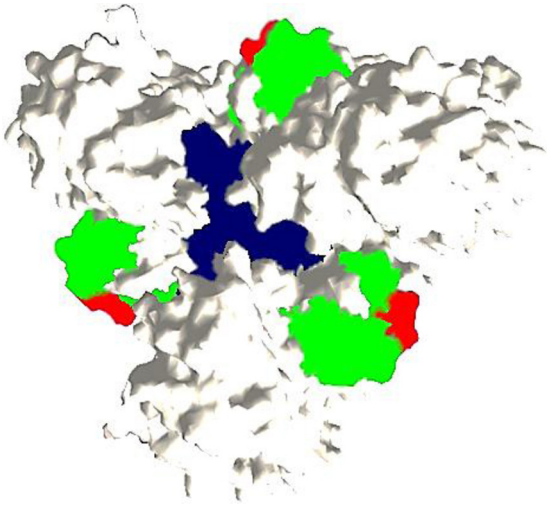

Fig. 3. Location of the gpG_{VHSV} major inducer peptides of type I INF responses and the RGD motif onto the top surface of the pre-fusion conformation trimer. Dark blue, p31 (residues 280–310); green, p33 (residues 340–370); purple, RGD motif (residues356–358) (Chico et al., 2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 1
Correspondence between gpG_{VSV} and gpG_{VHSV} amino acid numbering used in this review (Roche et al., 2006, 2007; Walker and Kongsuwan, 1999).

Domain	characteristics	gpG_{VSV}	gpG_{VHSV}
I	Receptor-binding	1–17	39–56
		311–382	342–405
II	α-helixes	25–28	65–70
		258–305	290–336
		384–405	407–428
III	Pleckstrin homology domain	37–46	79–88
		181–252	218*–290
IV	Fusion domain	52–180	94–218*

The published alignement between gpG_{VSV} and gpG_{VHSV} (Roche et al., 2006, 2007; Walker and Kongsuwan, 1999) were used. The gpG_{VSV} was traditionally numbered without the signal peptide, while the gpG_{VHSV} was numbered by considering the signal peptide as used before (Estepa et al., 2001). Their correspondances were tabulated for clarity. *, aproximated location due to an 8 amino acid gap required for maximal alignement. Most probably, for many of the precise locations mapped in the gpG_{VHSV} there are, at least, ±5 amino acid error and do not necessarily would apply to any other fish or mammalian rhabdoviral gpG. Important pepscan mapped locations in the gpG_{VHSV} were PS/PIPP-binding p2 (82–109)(Estepa and Coll, 1996), frg11 (56–110) (Estepa et al., 2001), MAb binding lineal epitopes (139–153 and 399–413)(Fernandez-Alonso et al., 1998), type I IFN inducing regions (280–310 and 340–370) (Chico et al., 2010) and autophagy-inducing regions (99–113)(Garcia-Valtanen et al., 2014). The tridimensional (3D) structure of the gpG_{VHSV} was modelled by using the above mentioned alignement and the Swiss–Model server (http://spdbv.vital-it.ch/) through the RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do), gpG_{VSV} files: 2CMZ.pdb (low pH) and 2J6J.pdb (physiological pH)(Roche et al., 2006, 2007).

well as of other gpGs (i.e.: gpG_{VHSV}) could be approximated by modeling after amino acid alignment (Falco et al., 2011; Walker and Kongsuwan, 1999).

The gpG of rhabdoviruses triggers membrane fusion by causing extensive low pH induced reversible conformational changes at its globular head (Mas et al., 2002). The different conformations are in equilibrium at the different intermediate pHs. Thus, in the physiological pH pre-fusion conformation, the gpG_{VSV} (88 Å) has the shape of a tripod with the antigenic sites located at the globular heads and the fusion domains pointing toward the viral membrane. Each fusion domain is made up of a segment folded in 2 fusion loops cross-linked by cysteine and located to the opposite end to the globular heads. In the low-pH post-fusion conformation the gpG becomes elongated to 125 Å, displaying a highly conformational changed globular head (domains I, II, and III) of about 60 Å of diameter with its unchanged but translocated fusion domains (domain IV) now in the opposite direction penetrating the cellular membrane (Fig. 2). The globular head folds into domains I, II, and III (Fig. 3): I) A sheet-rich lateral domain at the top of the gpG at physiological pH, II) A central, mostly α-helical domain responsible for trimerization of the top of gpG, and III) A neck which has the characteristic fold of pleckstrin homology domains. These compact domains are, nevertheless made up from discontinuous segments of the gpG (Table 1). Domain IV is inserted into domain III which is inserted into domain II. In both low and physiological pH conformations, the C-terminal part corresponding to the transmembrane domain and the fusion domains are located at the same end of the gpG molecule.

Conformational changes induced by low pH included most of the globular head domains I, II and III (Roche et al., 2006, 2007). At this respect, unlike many surface glycoproteins from other enveloped viruses, rhabdoviral gpG did not contain hydrophobic heptad repeats (3–4 contiguous sequences of 7 amino acids: abcdefg, hydrophobic amino acids in bold) forming amphipathic alpha helixes and coiled coils polymers related to viral fusion (Skehel and Wiley, 1998). Although, no coiled-coil motifs were predicted in the gpG of any rhabdovirus (Yao et al., 2003), after arbitrarily considering in positions a–d all the hydrophobic amino acids, non-canonical 2–3 hydrophobic heptad repeats could be detected in the gpG sequences of all rhabdoviruses in similar locations (Coll, 1995). Even though these non-cannonical predicted heptad repeats contain some proline residues (a residue which is normally precluded in amphipathic α-helix structures), there is a low-pH dependent–elongation on the modelled gpG_{VHSV} (Falco et al., 2011) corresponding to an α-helix domain II which is also one of the predicted 288–319 heptad repeats on that domain (Roche et al., 2006, 2007).

2.1. Identification of gpG peptides inducing type I interferon lead to novel DNA vaccine adjuvants

Type I interferon (IFN) is amongst the first line of defences against viral infections including those against rhabdoviruses. Type I IFN are a group of cytokines whose induction provokes the transcription activation of hundreds of genes (the interferon stimulated genes, ISG) e ncoding defensive host effector proteins (i.e.: Mx proteins) that control viral replication and restricting their spread to neighbouring cells (Stark et al., 1998). Although, the induction of type I IFN are mostly caused by viral genomes and/or replicative DNA/RNA intermediates, viral proteins have been also identified as strong type I IFN elicitors (Chico et al., 2010; Fitzgerald-Bocarsly, 1993; Ito et al., 1994; Miller and Anders, 2003; Seeds et al., 2006). For instance, a direct role of gpG on the induction of type I IFN has been shown with VHSV and infectious haematopoietic necrosis (IHNV) by using the corresponding gpG encoding plasmids (Boudinot et al., 1998; Chico et al., 2009; Kim et al., 2000; McLauchlan et al., 2003; Tafalla et al., 2008). Moreover, the gpG_{VHSV} protein on the surface of the transfected cells rather than that of gpG transcripts was more important in the induction of type I IFN (Acosta et al., 2006).

To investigate whether or not there were gpG_{VHSV} linear determinants triggering induction of type I IFN, a collection of overlapping 20-mer gpG_{VHSV} peptides were used to assay for induction of *mx3* transcription, one of the most characteristic type I IFN-stimulated genes (Chico et al., 2010). The results identified the 280–310 (p31 peptide) and 340–370 (p33 peptide) gpG_{VHSV} regions as peptide inducers of type I IFN responses. Both regions were located at the surface of the globular head of the gpG_{VHSV} molecule, according to the prefusion 3D modelled structure (Roche et al., 2006, 2007)(Fig. 3). The p31 peptide included the small peptide 299–313, earlier mapped as one of the T-cell gpG_{VHSV} epitopes identified in anamnestic immunoproliferative responses of leucocytes from trout surviving VHSV infections (Lorenzo et al., 1995). Similar locations were found for mice immunoproliferative peptides identified in gpG_{RV} (MacFarlan et al., 1984) or gpG_{VSV} (Burkhart et al., 1994). The p33 peptide included the ^{356}Arg-Gly-Asp ^{358}(RGD) motif (Fig. 3, red), an integrin recognition sequence (Ruoslahti, 1996; Villard et al., 2006), which many viruses use as receptors/co-receptors (Cseke et al., 2009).

With the aim to investigate whether or not the gpG_{VHSV} p31 and p33 peptides could be used as possible DNA vaccine adjuvants, their corresponding cDNA sequences flanked by the signal peptide and transmembrane sequences of gpG_{VHSV} were cloned into an eukaryotic expression cassette in a plasmid vector under the control of the cytomegalovirus promoter (pMCV1.4). The peptides would be anchored to the membrane of the cell, exposing the IFN-inducing peptides to the extracellular space In addition, a similar plasmid encoding both p31 and p33 peptide cDNA sequences linked by 6 glycines was also constructed (pMCV1.4-p31 + p33) (Fig. 4). Each of the pMCV1.4 plasmid constructs were then assayed for possible adjuvant activity by co-immunizing zebrafish with a DNA vaccine to spring viremia carp virus (SVCV) made in a fish expression plasmid under the control of a carp promoter (pAE6-gpG_{SVCV}) (Martinez-Lopez et al., 2014). The protection rates recorded after a lethal SVCV challenge in zebrafish immunized with a sub-protective dosage of pAE6-gpG_{SVCV} in combination with pMCV1.4-p31–p33 were higher than those conferred by the immunization with the vaccine alone. Furthermore, the combination of vaccine and adjuvant allowed a ~10-fold reduction of the vaccine dose without affecting its efficacy, reaching a 60-day survival rate of 70%. Therefore, the results showed that the co-injection of the SVCV DNA vaccine and the gpG_{VHSV} peptide-derived adjuvants allowed: (i) a ten-fold reduction in the dose of pAE6-Gsvcv without compromising its efficacy, (ii) an increase in the duration of protection, and (iii) an increase in the survival rate. This first report in which type I IFN-inducing peptides from a rhabdoviral gpG had been used to improve DNA vaccines against other virus, opened the door to search for a possible new generation of vaccine adjuvants based on viral components rather than bacterial LPS or host immune system molecules.

2.2. Identification of gpG peptides inducing autophagy might lead to develop novel therapeutic molecules

Autophagy is an evolutionarily conserved membrane-trafficking pathway to destroy self proteins that operates at low basal levels under normal conditions. Autophagy levels are raised in response against infections, intervening in the clearance of viruses (Klionsky et al., 2011; Tovilovic et al., 2014). Many authors have proposed that strategies aimed at modulating autophagy could be used in the prevention and treatment of infectious diseases. In this context, it was interesting to test if peptide-derived regions of gpGs might suffice to initiate an autophagy antiviral response. If that were the case, those peptides could be of interest for the development of antiviral agents.

The implication of the gpG_{VSV} protein in autophagy was first demonstrated in *Drosophila* by using ultraviolet-inactivated vesicular stomatitis virus (VSV) or gpGVSV-containing vesicles (Shelly et al., 2009). With the aim of identifying potential gpG autophagy-inducing peptides, sets of 15-mer overlapping peptides (pepscan) spanning gpG_{VHSV} and gpG_{VSV} were scanned for induction of LC3 (an usual protein marker for autophagy) by flow cytometry in human keratinocytes, the HaCaT cell line (gpG_{VSV}) and ZF4 (gpG_{VHSV}) cells (Garcia-Valtanen et al., 2014). Two autophagy-inducing regions located at the fusion (Fig. 5, blue) and/or the globular (Fig. 5, red) domains in gpG_{VSV} or gpG_{VHSV} were identified, suggesting a key role of part of the fusion domain not only in phospholipid binding as previously described (Estepa et al., 2001 Nunez et al., 1998) but also in autophagy. In this respect, it was interesting to note that PIPP/PI play an important role in the autophagy processes (Criollo et al., 2007; Rong et al., 2012). The autophagy-inducing regions located at the fusion domain in gpG_{VSV} (77–91) or gpG_{VHSV} (99–113) (Fig. 5, blue) were defined by one peptide, which increased >3-fold the LC3-fluorescence. The second region (337–361) that only appeared in gpG_{VSV} was defined by two pepscan peptides (Fig. 5 VSV). These results suggest a pivotal role of the fusion domain of gpGs in autophagy (Garcia-Valtanen et al., 2014). The role in autophagy of the gpG_{VHSV} and gpG_{VSV} inducing-peptides mapped by pepscan was confirmed by induction of conversion between LC3-I to LC3-II (the lipidated form of LC3) assayed by Western blotting. In the case of gpG_{VHSV}, the results were also confirmed by using a previously identified peptide, p2, described as the major phospholipid binding domain of gpG_{VHSV}, is included in the autophagy-inducing region located at the fusion domain. Furthermore, the autophagy inducing peptides belonging to the fusion domains, reduced ~10-fold the viral *in vitro* titers of VHSV or SVCV (Garcia-Valtanen et al., 2014). Therefore, probabilities are good that p2-derived regions of gpG_{VHSV} could be used to initiate defensive autophagy *in vivo* responses to improve protection, even once the VHSV infection has been initiated. However, that still remains to be proven.

3. Conclusions

We have reviewed here some of the novel examples which focusing on gpG_{VHSV} show that the pepscan technique could be successfully used to identify single peptides of 15–20 mer responsible for: (i) triggering type I IFN early events of host innate immune response (Chico et al., 2010) and (ii) initiating antiviral autophagy

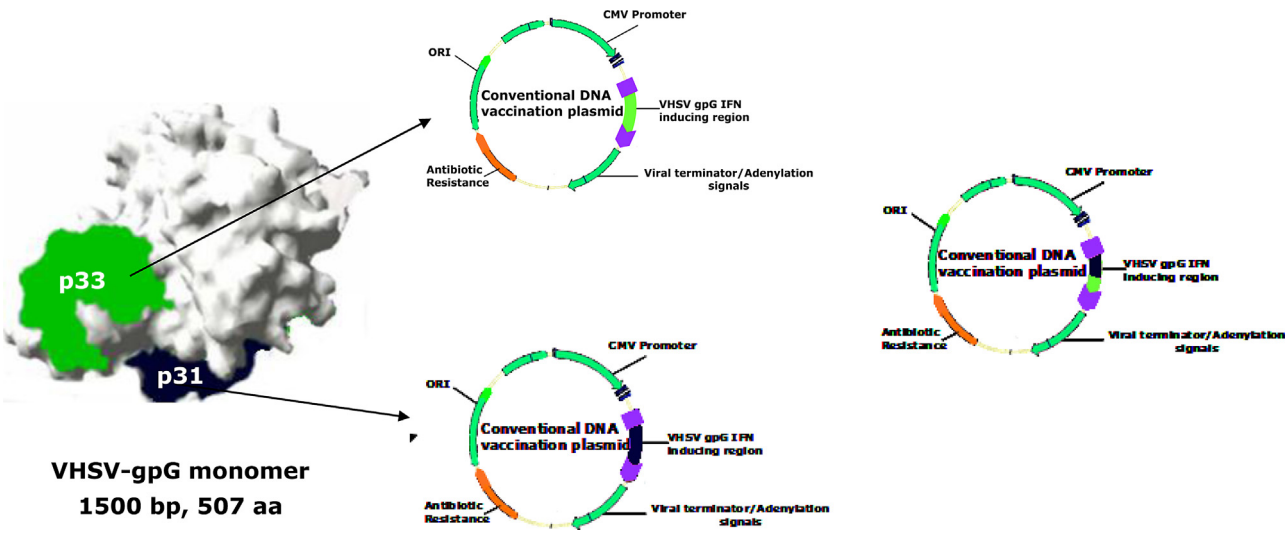

Fig. 4. Location of the major inducer peptides of type I INF responses in the of gpG_{VHSV} monomer and scheme of the DNA plasmids encoding their cDNA sequences. The plasmids contain the cytomegalovirus promoter region (CMV) upstream of the signal peptide of gpG_{VHSV} (SPVHSV) followed by the cDNA sequences of p31, p33 or p31 + p33 (linked by six glycines) and the transmembrane domain (TMVHSV) of of gpG_{VHSV}.

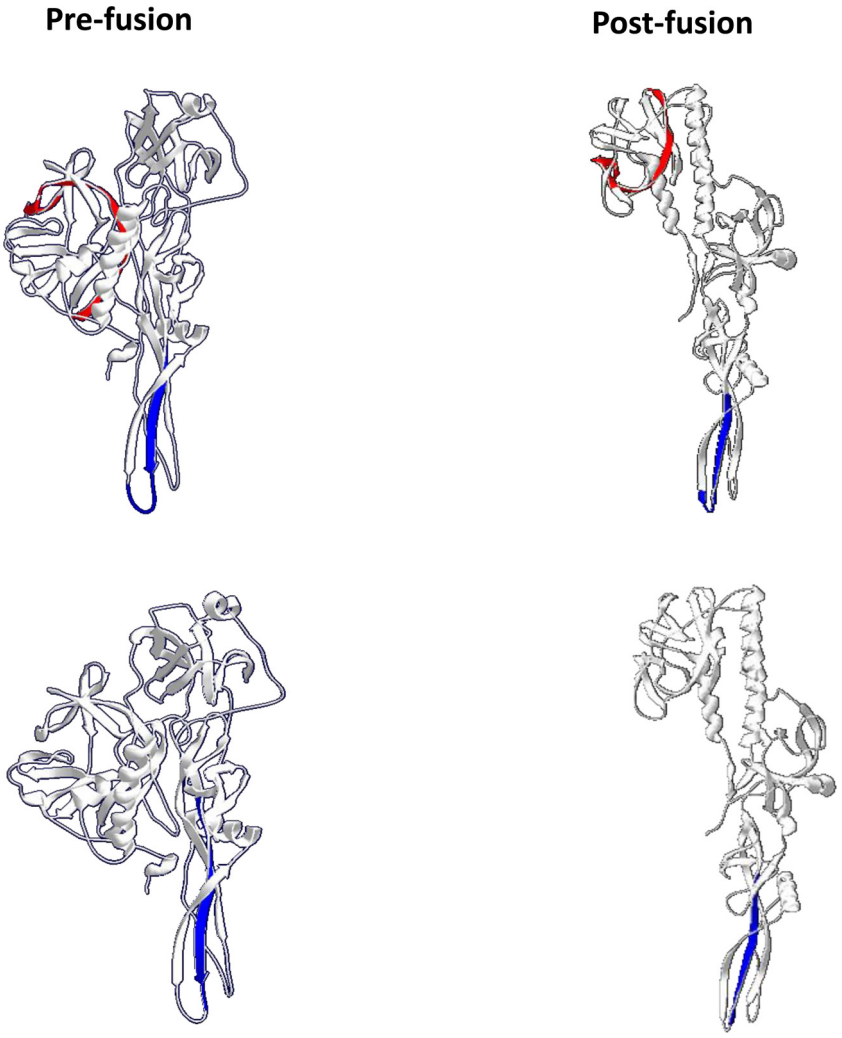

Fig. 5. Location of the autophagy-inducing peptides in the of gpG_{VHSV} monomer. The gpG_{VSV} (77–91 blue and 337–361 red) and gpG_{VHSV} (99 113 blue) major autophagy inducing peptides were mapped onto the pre-fusion and post-fusion conformations of the corresponding gpG trimers (Garcia-Valtanen et al., 2014). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

(Garcia-Valtanen et al., 2014). Previous work showed the importance of similar short peptides for virus-host membrane fusion (Estepa et al., 2001), host anamnestic immune responses (Lorenzo et al., 1995), and mapping of monoclonal antibodies (MAbs) recognizing linear epitopes (Fernandez-Alonso et al., 1998). Although the pepscan strategy does not allow to detect conformational functionalities, it was a highly useful technique which allowed the screening of the whole sequence of rhabdoviral gpG_{VHSV} by means of 15–20 mer peptides, the identification of minimal determinants for several gpG_{VHSV} functionalities and the complementation with further biotechnological techniques to devise novel applications to fight aquatic rhabdovirosis including now the design of lineal antigens for improvements on DNA vaccines, or novel adjuvants that decrease DNA vaccine requirements, and the identification of innovative therapeutic molecules.

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