Fine mapping of a salmonid E2 alphavirus neutralizing epitope

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In this study, we aimed to characterize the epitope recognized by the neutralizing 17H23 mAb directed against the E2 glycoprotein of most of salmonid alphavirus (SAV) subtypes and widely used in several laboratories to routinely diagnose SAV. We hypothesized that the 17H23 epitope was located in the major domain B, previously identified in the E2 of mammalian alphaviruses as the domain recognized by most of the E2 neutralizing mAbs. Indeed, the SAV E2 domain B counterpart is contained in the protein domain previously characterized as being recognized by mAb 17H23. Thus, to precisely characterize the 17H23 epitope, we developed an alanine scanning mutagenesis approach coupled with the generation of the respective recombinant SAV (rSAV) by using the available infectious cDNA. Ten mutant rSAVs termed A–J from E2 aa 223–236 were produced and characterized in vitro using indirect immunofluorescence assays on virus-infected cells with mAbs 17H23, 51B8 (another non-neutralizing anti-E2 mAb) and 19F3 directed against the non-structural protein nsp1. Two of the mutant rSAVs (G and H) escaped neutralization by mAb 17H23. In addition, we showed that when juvenile trout were infected by bath immersion with the rSAV mutants, some of them were either totally (D, E and G) or partially (H) attenuated. Together, the data from the in vitro and in vivo experiments indicated that the putative 17H23 amino acid sequence epitope comprised the short amino acid sequence 227 FTSDS 231.

INTRODUCTION

Sleeping disease in trout was first observed in France in 1985 (Anonymous, 1985). Disease in rainbow trout is characterized by the abnormal behaviour of the fish, which stay on their side at the bottom of the tanks, reminiscent of a ‘sleeping state’, and thus giving the terminology of sleeping disease (Boucher & Baudin-Laurencin, 1994). The lesion responsible for this behaviour was presumed to be extensive necrosis of the skeletal red muscle. This chronic stage follows characteristic sequential histological lesions of the exocrine pancreas and heart (Boucher & Baudin-Laurencin, 1996; McLoughlin & Graham, 2007; Biacchesi et al., 2015). Rainbow trout at all stages of production may be affected by this disease. Although non-officially accepted by the International Committee on Taxonomy of Viruses, alphaviruses affecting salmonid are now termed salmonid alphavirus (SAV). Three main subtypes are recognized: SAV1 is the salmon pancreas disease virus causing pancreas disease in salmon in Ireland and Scotland, SAV2 is the sleeping disease virus causing sleeping disease in freshwater rainbow trout, and SAV3 is the Norwegian salmon virus causing pancreas disease in salmon and marine rainbow trout in Norway. Some studies now suggest that there may be up to six different subtypes of the virus (Fringuelli et al., 2008). In recent years, SAV has becoming more and more predominant in the field and there is now a real challenge to eradicate this pathogen in most fish farms (Mérou & Brémont, 2015). These viruses have been shown to spread efficiently horizontally via direct water-borne transmission (McLoughlin et al., 1996). The possibility of vertical transmission for SAV has been suggested (Bratland & Nylund, 2009) but not yet clearly demonstrated (Kongtorp et al., 2010). As for all alphaviruses, SAV genomes consist of a positive-sense ssRNA molecule of roughly 12 kb encoding two polyproteins, which, after processing through proteolytic cleavages, produce the viral mature products: the non-structural proteins nsP1, nsP2, nsP3 and nsP4, which altogether constitute the replicase complex, and the major structural proteins of the capsid (C) and the two external glycoproteins (E2 and E1). Three main features distinguish SAV from mammalian alphaviruses: (i) SAV isolates are very close phylogenetically but only distantly related to the other alphaviruses; (ii) SAV non-structural and structural proteins are larger than those of mammalian alphaviruses; and (iii) arthropod-independent virus transmission to the host has been demonstrated in cohabitation experiments (Boucher, 1995), a phenomenon that has never been
documented for mammalian alphaviruses. In a previous study, the protein domains recognized by a panel of mAbs directed against some of the non-structural and structural proteins of SAV2 were roughly mapped (Moriette et al., 2005). In the current study, we aimed to define and characterize the epitope recognized by the neutralizing 17H23 mAb directed against the E2 glycoprotein of most of SAV isolates and widely used in a number of laboratories to routinely diagnose SAV. We hypothesized that the 17H23 epitope is located in the major domain B identified previously in the E2 of mammalian alphaviruses as the domain recognized by most of the E2 neutralizing mAbs (Voss et al., 2010; Fox et al., 2015). Indeed, the SAV E2 domain B counterpart is contained in the protein domain previously characterized as being recognized by mAb 17H23 (Moriette et al., 2005). Thus, to precisely characterize the 17H23 epitope, we developed an alanine scanning mutagenesis approach coupled with the generation of the respective recombinant SAV2 (rSAV) using the available infectious cDNA (Moriette et al., 2006; Mérour et al., 2013). Most of the mutant rSAVs were recovered and characterized in vitro, and in vivo by their phenotypes. Together, the results allowed the determination of a short motif of 5 aa as being the 17H23 epitope.

RESULTS

Generation of mutated rSAV by alanine scanning

To produce rSAV with targeted mutations in the E2 glycoprotein, a BrsGI DNA fragment cloned in the pJet1.2 plasmid (Mérour et al., 2013; Fig. S1, available in the online Supplementary Material), which covers the complete E2 sequence, was used as a template to introduce the desired mutations by site-directed mutagenesis. As presented in Table 1, 10 consecutive amino acid substitutions, termed mutant A–J, were introduced into the E2-derived domain B to replace each amino acid by an alanine. Each mutation was confirmed by nucleotide sequencing with two primers, MutSeqF and MutSeqR (Table 1). Each of the mutated BrsGI DNA inserts was introduced back into the pSAV infectious cDNA backbone (Fig. S1). Plasmids pSAV mutants A–J were introduced via electroporation into BF-2 cells to recover the corresponding rSAV. Ten days later, the supernatants containing rSAV were used to infect fresh BF-2 cells.

Characterization of the recovered rSAV in cell culture

Rescue of the expected mutant rSAV was ascertained by immunofluorescence assays on BF-2 cells infected by WT SAV and the mutant rSAV. Fig. 1 shows the results of the immunofluorescence assays with the anti-nsP1 mAb 19F3 or the non-neutralizing anti-E2 mAb 51B8, respectively, conducted on BF-2 cells infected with the supernatants from cells electroporated with the various mutagenized pSAVs. With the exception of mutant J, all mutated rSAVs were readily rescued (Fig. 1). Cells infected with the rSAV mutants were also subjected to immunofluorescence assays using the neutralizing anti-E2 17H23 mAb. The data are presented in Table 2, indicating that some of the rSAV mutants, such as F and G, were not recognized by 17H23 mAb, although they were recognized by the non-neutralizing anti-E2 mAb 51B8. Cells infected with the supernatant from the cells electroporated with the pSAV F mutant exhibited a very low number of positively infected cells in the immunofluorescence assay, reflecting a very low efficiency in the recovery of that mutant. This observation suggested that the change of the S229 residue to A had a drastic effect on the rSAV F mutant replication cycle. The virus titre of the rSAV F mutant at the first passage in cell culture was only 10^5 p.f.u. ml^-1 compared with 10^7 p.f.u. ml^-1 for the WT SAV (data not shown). Mutant F was lost after two passages in cell culture. After resequencing of this mutant, we observed that an incidental mutation had been introduced: the T226 residue had been changed to A (codon ACC converted to GCC), as for mutant E. In mutant E, the change of T226 to A did not induce any phenotype modification in cell culture. We can assume therefore that the S229 residue is absolutely required for efficient growth in cell culture. Mutant J could not be recovered when the C235 residue was replaced by an A. This Cys residue, conserved in all E2 alphaviruses including SAV, is involved in a disulfide bond (Voss et al., 2010; Snyder et al., 2012), which was destroyed by the alanine replacement, destabilizing the three-dimensional structure of SAV E2. For the others, virus titres after two passages in cell culture ranged from 5 x 10^5 to 2 x 10^7 p.f.u. ml^-1 (Table 2). Reverse transcription (RT)-PCR on genomic RNA extracted from rSAV aliquots obtained after two passages in cell culture were achieved with primers annealing in the E2 domain B sequence (Table 2). Gel-purified PCR products were sequenced, thus confirming the presence of the targeted mutation.

Replication kinetics of the mutant rSAV

The kinetics of replication of each mutant rSAV was evaluated on rSAV-infected cell supernatants collected at passage 2. All the mutant rSAVs grew well in cell culture without presenting an attenuated phenotype. Their kinetics of replication were comparable to that of the WT SAV (Fig. 2). The final titre at 10 days post-infection (p.i.) ranged from 10^6 (mutants D and G) to 10^8 (mutant H) p.f.u. ml^-1, respectively. All the other rSAV mutants reached titres around 10^7 p.f.u. ml^-1. No drastic impact of the mutation on the kinetics of replication for any of the mutant rSAVs was observed in vitro in cell culture.

Seroneutralization assays on rSAV

To evaluate the impact of the various mutations introduced by alanine scanning in the E2 B domain on the
recognition by neutralizing anti-E2 17H23 mAb, seroneutralization assays were performed. All the mutant rSAVs and WT SAV were incubated with mAb 17H23 as described in Methods, before being used to infect BF-2 cells. Infection of BF-2 cells by the various rSAV mutants was visualized by immunofluorescence assays as detailed above. Fig. 3 shows that, with the exception of two rSAV mutants, G and H, all the other rSAVs and WT SAV were neutralized by mAb 17H23. For mutants G and H, the replacement of the two successive D230 and S231 amino acids allowed the mutants to escape neutralization by mAb 17H23, suggesting that these two residues are part of the 17H23 epitope (Table 2). As already mentioned above, while mutant G was not recognized by mAb 17H23, mutant H was recognized but exhibited a different immunofluorescence staining pattern (Fig. S2).

Table 1. Primer sequences used in the study

<table>
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<th>Primer name</th>
<th>Primer sequence (5’→3’)†</th>
<th>Amino acid change</th>
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<tr>
<td>BsrGIR</td>
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<tr>
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<tr>
<td>MutSeqR</td>
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</tr>
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<td>Mut A</td>
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<td>Mut B</td>
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<tr>
<td>Mut C</td>
<td>CCGTGCAAGAGGCGTACCTTCACCC</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>D→A</td>
</tr>
<tr>
<td>Mut H</td>
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<tr>
<td>Mut I</td>
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<td>Mut J</td>
<td>CTCCAGAGGAGGACTCCAGGAGTTACG</td>
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*Codon changes are underlined.

Fig. 1. Immunofluorescence assays on rSAV-infected cells. BF-2 cells were infected with the various rSAVs (A–I) or the WT SAV. At 6 days post-infection (p.i.), the cells were fixed and stained by immunofluorescence with SAV anti-nsP1 mAb 19F3 (a) or anti-E2 mAb 51B8 (b). Mock, mock-infected cells.
Fish infections with the mutant rSAVs

The kinetics of replication of the mutant rSAVs indicated that none of these mutants was attenuated in vitro, so it was of interest to evaluate whether some mutant rSAVs had been attenuated in vivo following the amino acid changes. For this, 50 virus-free juvenile rainbow trout (mean weight 1.5 g) were infected by bath immersion in tanks filled with 3 l of freshwater, with rSAVs at a final titre of $5 \times 10^4$ p.f.u. ml$^{-1}$ for 2 h at 10°C. Controls were fish that were mock infected with serum-free culture medium under the same conditions. Mortalities were recorded over a 2-month period. Fig. 4 illustrates that some of the mutant rSAVs such as mutants D, E and G were almost totally attenuated in trout with a 98% survival rate, while mutant H was only slightly attenuated (56% survival rate) and mutant C was as virulent as the WT SAV (46% survival rate). The other mutants, A, B and I, were phenotypically similar to the WT SAV in fish (data not shown).

<table>
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<th>E2 antibody (51B8)</th>
<th>E2 neutralizing antibody (17H23)</th>
<th>Seroneutralization assay</th>
<th>Titre (p.f.u. ml$^{-1}$)</th>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>$5 \times 10^7$</td>
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ND, Not done; +, positive staining; −, negative staining.
*Mutant F was lost after two passages in cell culture.
†Different staining compared with that of WT.

**Table 2.** rSAV mutant characterization

**Fig. 2.** Kinetics of replication of the mutant rSAVs. BF-2 cells were infected with the various rSAVs (MutA–I) or the WT SAV at an m.o.i. of 0.1. At the indicated times post-infection (p.i.), cell supernatant was collected and virus was titrated as described in Methods.
(2.3 × 10⁹ p.f.u. ml⁻¹). At day 15, with the exception of mutant D in which only one fish out of four was infected, three or all four of the fish collected after infection in the other groups with the rSAVs tested positive. At day 21, all the infected groups were positive for rSAV, although the number of positive fish in each group was variable. These data confirmed that all the rSAVs were able to enter and to replicate in fish.

**DISCUSSION**

With the aim of precisely characterizing the 17H23 neutralizing epitope directed against the E2 glycoprotein of most SAVs, we developed an alanine scanning mutagenesis approach coupled with the generation of the respective rSAVs by using the available infectious cDNA (Moriette et al., 2006). For mammalian alphaviruses, an E2-derived domain B has been shown to be the major target for neutralizing mAbs (Porta et al., 2014; Fox et al., 2015). In a previous study, the domain recognized by mAb 17H23 on the SAV E2 glycoprotein was roughly mapped and included the SAV E2 domain B counterpart (Moriette et al., 2005). In this alanine scanning approach, 10 amino acid changes from E223 to C236 were introduced by site-directed mutagenesis in the SAV infectious cDNA. Eight viable mutants rSAV were recovered and amplified; however, as expected, the replacement of the C236 by
alanine profoundly affected the correct folding of the E2 glycoprotein and made this mutation lethal. Interestingly, another mutant, mutant F, in which S229 was exchanged by alanine had a deleterious effect. The rSAV mutant F could be recovered but was lost after two passages in cell culture. Thus, S229 seems to play a crucial role either in the E2 structure or in the E2–E1 interaction. It must be emphasized that when S229 is replaced by a conservative residue such as threonine, the SAV is viable (Fringuelli et al., 2008). A three-dimensional model based on the Chikungunya E2–E1 X-ray crystallography structure (Voss et al., 2010) showed that serine and the surrounding amino acid residues are located in an external loop (Fig. S3A). Data from the in vitro experiments together with the in vivo experimental fish infections provided some clues on the amino acid residues constituting the 17H23 neutralizing epitope. Among the rSAV mutants that were engineered and recovered, two – mutants G (D230A) and H (S231A) – were able to escape neutralization by mAb 17H23, meaning that both D230 and S231 are key residues in the epitope recognized by this mAb. Furthermore, mutant G was almost totally attenuated in trout, inducing only 2% of cumulative mortality in infected fish, while mutant H was partially attenuated (44% cumulative mortality). Virus escape mutants are generally attenuated in the host compared with WT virus (see Pal et al., 2014, for an example in alphaviruses). Interestingly, mutant F (S229A) could be rescued but was lost after two passages.

![Graph showing virus titres in sera from mutant rSAV-infected fish at 15 and 21 days post-infection. Four or three infected fish were harvested randomly in each group at days 15 and 21 post-infection. The virus titre for each fish was then determined by immunofluorescence assay from the serum. The mean virus titres ± SE for each group are shown, together with the number of fish positive for virus detection. The limit of detection was 10^-3 p.f.u. ml^-1.](image-url)
in cell culture, emphasizing that this residue, adjacent to D230, probably plays a major role in SAV E2 conformation and/or function. Mutants D and E (F227A and T228A, respectively) were neutralized by mAb 17H23 but exhibited an interesting phenotype in vivo, as both rSAV mutants were fully attenuated in trout with no mortality recorded during the 2 months of the experimental fish infection trial. It was shown that these mutants replicated in fish at titres ranging from $1.3 \times 10^6$ up to $2.5 \times 10^9$ p.f.u. ml$^{-1}$, similar to the WT SAV ($2.3 \times 10^6$ p.f.u. ml$^{-1}$), but were indeed fully attenuated. The other mutants, A, B and I, were neutralized by mAb 17H23 and were phenotypically similar to the WT SAV in terms of kinetics of replication and final titre, indicating that the corresponding amino acid residues were not part of the 17H23 epitope and do not play a major role in SAV E2 conformation or in SAV binding to the cell surface. Thus, the short amino acid sequence containing the 17H23 epitope is localized in a region, conserved in all mammalian alphaviruses, that has been shown to be the main E2 protein domain for recognition by neutralizing mAbs (Fox et al., 2015; Fig. S3B).

**METHODS**

**Cells and virus.** Bluegill fry (BF-2) cells were cultured in Galloway's minimal essential medium/25 mM HEPES (GMEM/HEPES; Eurobio) supplemented with 10% FBS (Eurobio), 1% phosphate tryptose broth (Sigma), 2 mM L-glutamine (PAA) and penicillin (100 U ml$^{-1}$) supplemented with 10% FBS (Eurobio), 1% phosphate tryptose broth and streptomycin (100 μg ml$^{-1}$) (BIOVALLEY). SAVs were grown in fresh BF-2 cells in 24-well plates in serum-free GMEM/HEPES and incubated at 10°C in an incubator without CO$_2$.

**Generation of mutated pSAV constructs by alanine scanning mutagenesis.** From the pSAV infectious plasmid construct containing the full-length SDV cDNA as template (Mori et al., 2006; Fig. S1), a BrSGI DNA fragment encompassing the complete E2 coding region was amplified by PCR using the BrsGIF and BrsGIR primers (Table 1) and cloned into the pET12 vector (ThermoFisher Scientific). Alanine scanning on the E2 domain B was achieved by site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's recommendations and the primers MutA to MutJ (Table 1). All the targeted mutations were verified by nucleotide sequencing. Each of the mutated BrSGI DNA fragments was cloned back into the BrsGI-digested pSDV backbone.

**Recovery of rSAV from mutated pSAV constructs.** Approximately $5 \times 10^6$ BF-2 cells per well were grown in six-well plates and transfected by electroporation (AMAXA Biosystems) with 2 μg each of the mutated pSAV constructs. Cells were incubated at 20°C overnight in 10% fresh medium. At 24 h post-transfection, the medium was replaced with serum-free fresh medium and the cells were incubated at 10°C for 6–10 days. The mutant rSAVs were amplified through two passages on fresh BF-2 cells. Viruses were titrated by immunofluorescent assays as described below.

**Virus titration and multi-step growth curves.** Virus titration for all the mutant rSAVs was achieved as follows: supernatants from rSAV-infected cells at an m.o.i. of 0.1 were collected from 2 to 10 days p.i. Tenfold dilutions of the collected supernatants were used to infect in duplicate BF-2 cells grown in 96-well plates. At 10 days p.i., virus titre was estimated after indirect immunofluorescence assays as described below.

**RT-PCR on rSAVs.** Supernatant aliquots of rSAV-infected BF-2 cells were clarified, and genomic viral RNA was extracted using a QIAamp viral RNA Purification kit (Qiagen) according to the manufacturer's recommendations. Part of the RNA genome was amplified by RT-PCR with specific primers in the E2 region: BrsGIR for reverse transcription and MutSeqF and MutSeqR for PCR (Table 1, Fig. S1). PCR products were agarose gel purified and subjected to nucleotide sequencing.

**Indirect immunofluorescence assays.** rSAV-infected BF-2 cells were fixed at 6 days p.i. with a mixture of ethanol:acetone (1:1, v/v) for 20 min at −20°C and then air dried. Immunofluorescence assays were achieved using various mAbs directed against either SAV nsP1 protein (mAb 19F3) or against SAV E2 protein (17H23, a neutralizing mAb, and 51B8, a non-neutralizing mAb). Fixed BF-2 cells were incubated for 45 min at room temperature with primary mouse mAb 19F3 diluted 1:10000, 17H23 diluted 1:10000 or 51B8 diluted 1:500 in PBS with Tween 0.05% (PBS-T), and washed three times in PBS-T. The cells were then incubated for 45 min at room temperature with FITC-conjugated sheep anti-mouse antibody (P.A.R.I.S.) diluted 1:400 in PBS-T and washed three times in PBS-T. Positive infected cell foci were counted under a UV-light microscope (Nikon).

**Seroneutralization assays.** BF-2 cells were grown in 24-well plates and incubated at 20°C overnight in 10% fresh medium. The amount of each rSAV corresponding to an m.o.i. of 0.1 was then incubated with neutralizing mAb 17H23 diluted 1:5000 (1:1, v/v) for 1 h at 10°C in serum-free medium and a total volume of 200 μl was used to infect BF-2 cells for 1 h at 10°C. The inoculum was removed, serum-free medium was added and the cells were incubated for 6 days at 10°C. The cells were fixed and immunofluorescence assays were carried out using anti-SAV nsP1 mAb 19F3 and anti-SAV E2 mAb 51B8. Observations were done under a UV-light microscope.

**Experimental fish infections with the mutant rSAVs.** All animal experimental procedures were approved by the local ethics committee on animal experimentation: COMETHEA INRA under permit number 12/111. Fifty virus-free juvenile rainbow trout (Oncorhyncus mykiss; mean weight 1.5 g) were infected by bath immersion in tanks filled in with 3 l fresh water with rSAVs at a final titre of $5 \times 10^9$ p.f.u. ml$^{-1}$ for 2 h at 10°C. The tanks were then filled up to 30 l with fresh water. Controls were fish mock infected with serum-free culture medium under the same conditions. Mortalities were recorded daily over a 2-month period.

**Virus isolation from rSAV-infected fish.** Virus isolation was performed from whole blood samples harvested at 15 and 21 days p.i. on three or four randomly selected fish per group. Briefly, whole blood was collected and allowed to clot and separate overnight at 4°C. After centrifugation at 1200 g for 15 min at 4°C, the serum was recovered. Finally, BF-2 cells were inoculated with the serum diluted from serial 10-fold dilutions and incubated at 10°C for 7 days. The mean virus titre for each group was then determined by immunofluorescence assays (see above).

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