Deletions in the highly polymorphic region (HPR) of infectious salmon anaemia virus HPR0 haemagglutinin–esterase enhance viral fusion and influence the interaction with the fusion protein

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Since the discovery of a non-virulent infectious salmon anaemia virus (ISAV) HPR0 variant, many studies have speculated on the functional role of deletions within the highly polymorphic region (HPR) of genomic segment 6, which codes for the haemagglutinin–esterase (HE) protein. To address this issue, mutant HE proteins with deletions in their HPR were generated from the Scottish HPR0 template (NWM10) and fusion-inducing activity was measured using lipid (octadecyl rhodamine B) and content mixing assays (firefly luciferase). Segment six HPR was found to have a strong influence on ISAV fusion, and deletions in this near-membrane region predominantly increased the fusion-inducing ability of the resulting HE proteins. The position and length of the HPR deletions were not significant factors, suggesting that they may affect fusion non-specifically. In comparison, the amino acid composition of the associated fusion (F) protein was a more crucial criterion. Antibody co-patching and confocal fluorescence demonstrated that the HE and F proteins were highly co-localized, forming defined clusters on the cell surface post-transfection. The binding of erythrocyte ghosts on the attachment protein caused a reduction in the percentage of co-localization, suggesting that ISAV fusion might be triggered through physical separation of the F and HE proteins. In this process, HPR deletion appeared to modulate and reduce the strength of interaction between the two glycoproteins, causing more F protein to be released and activated. This work provides a first insight into the mechanism of virulence acquisition through HPR deletion, with fusion enhancement acting as a major contributing factor.

INTRODUCTION

Infectious salmon anaemia is a viral disease of Atlantic salmon that was first recorded in Norway in 1984 (Thorud & Djuvpik, 1988) and has since been reported in North America, Scotland, the Faroe Islands and Chile (Bouchard et al., 1999; Godoy et al., 2008; Kibenge et al., 2001; Mullins et al., 1998; Scyth et al., 2003). This enveloped virus belongs to the family Orthomyxoviridae, which includes the influenza A, B and C viruses, Thogoto virus and Quaranjavirus (http://ictvonline.org/virusTaxonomy.asp). The virus genome is composed of eight negative-sense, single-stranded RNA segments (Mjaaland et al., 1997) coding for at least ten viral proteins. Segments 5 and 6 encode the two main surface glycoproteins, the fusion (F) and haemagglutinin–esterase (HE) proteins, respectively (Aspehaug et al., 2005; Falk et al., 2004; Krossøy et al., 2001; Rimstad et al., 2001).

A putative non-virulent variant of infectious salmon anaemia virus (ISAV) was first reported in Scotland in 2002 (Cunningham et al., 2002) and later in many other salmon-producing countries (Christiansen et al., 2011; Cook-Versloot et al., 2004; Kibenge et al., 2009; Nylund et al., 2007). These variants are characterized by a segment 6 carrying a ‘full-length’ highly polymorphic region (HPR), encompassing a 35 aa stretch, located in the near-membrane domain of the protein stalk (Cunningham et al., 2002; Devold et al., 2001; Mjaaland et al., 2002; Nylund et al., 2003). This atypical ISAV segment 6 gene sequence, the longest discovered to date, is often referred to as HPR0 (Cunningham et al., 2002; Nylund et al., 2003). Interestingly, all currently characterized pathogenic ISAV isolates (Kibenge et al., 2007; Nylund et al., 2003, 2007) have deletions in the HPR compared with HPR0 variants (Mjaaland et al., 2002). This led to the hypothesis.
that virulent strains may have arisen from a reservoir of non-pathogenic HPR0 through deletion events in the segment 6 HPR (Mjaaland et al., 2002). However, the functional role of the HPR and the deletions within it has yet to be elucidated.

The HE globular head harbours a receptor-binding site that allows ISAV to attach to 4-O-acetylated sialic acid cell-surface receptors, and has acetylcholinesterase activity that destroys the sialic acid bonds (Falk et al., 1997; Hellebø et al., 2004). The latter function may assist in the release of virus particles, prevent self-aggregation of newly formed virions and promote virus uptake by endocytosis (Ohuchi et al., 2006; Palese & Shaw, 2007). The ISAV F protein is a 50 kDa glycoprotein identified as a type I F protein, which requires proteolytic cleavage to become activated and deploy the fusion peptide (Aspehaug et al., 2005). Subsequent merging of the cell and virus membrane bilayer occurs in two steps. In the first, the two outer-lipid leaflets fuse together (White et al., 2008). This intermediate stage, referred to as hemifusion, has been quantified previously for several paramyxoviruses and orthomyxoviruses using fluorometric lipid mixing assays based on octadecyl rhodamine B (R18) labelling of erythrocytes (Hoekstra et al., 1984). In the second step, the F protein promotes the merging of the two inner lipid layers and the formation of pores, which allows a direct exchange between the cytoplasmic contents of the virus and target cell (Chang et al., 2008). In this study, cellular cytoplasmic mixing was investigated by adapting an erythrocyte ghost (EG) gene-delivery technique (Byun et al., 2004) and measuring luciferase expression.

The physical separation of the receptor attachment and fusion functions on two different proteins implies that ISAV follows a strategy more akin to that of some paramyxoviruses (Aspehaug et al., 2005; Falk et al., 2004). Studies on these viruses have demonstrated that receptor binding induces conformational changes in the attachment protein, which promote physical interaction with the F protein and are essential for its activation (Corey & Iorio, 2007; Lee et al., 2008; Melanson & Iorio, 2004; Plemper et al., 2011; Zimmer et al., 2005). More specifically, the near-membrane stalk domain of the haemagglutinin (H) protein has been identified as a site of interaction critical to the activation of the F protein (Brindley & Plemper, 2010; Lee et al., 2008; Melanson & Iorio, 2004; Zhou et al., 1997).

We previously demonstrated that HPR deletions did not affect the primary receptor-binding and destroying functions located in the globular head of the HE protein (McBeath et al., 2011). Therefore, the main objective of the present study was to investigate whether deletions in the segment 6 near-membrane HPR influenced the ISAV fusion process. The hypothesis was that deletion within the HPR of a ‘full-length’ HE protein would enhance the fusion-inducing ability of the resulting HE. To accomplish this, several HPR-deleted HE mutants were created from a ‘full-length’ HPR0 sequence (NWM10), based on known deletions reported from wild-type virulent ISAV strains.

These were chosen to provide a representative range of different HPR types (i.e. position and length) and co-expressed along with F proteins from two known pathogenic viruses (strain Nevis or Glesvaer). In addition, this study investigated how HPR deletions may directly influence the strength of interaction between the two surface glycoproteins and the activation of the F protein.

**RESULTS**

**Deletions within a ‘full-length’ HPR influence fusion activity**

The first approach taken was to study the impact of HPR deletions on fusion activity, both for the initial hemifusion (lipid mixing) and final stage of fusion (content mixing assay) (Fig. S2a, b, available in the online Supplementary Material). We prepared different mutated variants of HE (Fig. 1) from an HE0 reference protein (HPR0, NWM10) and combined these with two different fusion proteins, F-Gle or F-Nev, originating from two ISAV strains. Homotypic combinations of HE and F proteins were used as positive controls in each experiment (HE-Gle/F-Gle and HE-Nev/F-Nev) to ensure that heterotypic glycoproteins were capable of generating equivalent fusion levels. This indicated that the *in vitro* system developed was functionally reliable for assessing the effect of HPR deletion on the fusion process. Comparable levels of haemadsorbed EGs were observed between HE mutants throughout each experiment. HE0/F-Gle induced extremely low fusion activity, with the lipid mixing assay indicating 1 % hemifusion, and the content mixing assay showing a firefly luciferase (FF) to *Renilla* luciferase (RL) ratio (FF/RL) of 0.12. In contrast, all HPR deletions generated in mutant HE (Mut2–7/F-Gle in Fig. 1) significantly increased the fusion-inducing activity relative to HE0/F-Gle, both for the lipid and content mixing assays (Fig. 2a, b). Mut2/F-Gle (HPR7) produced the highest increases with 46.3 % hemifusion and 11.67 FF/RL, whereas Mut5/F-Gle (HPR8) resulted in a similarly high hemifusion level of 38 % but induced one of the lowest levels of content mixing at 1.65 FF/RL, still 14 times higher than those of the initial HE0/F-Gle. The artificial Mut8/F-Gle (33 aa deletion) induced an extremely low level of fusion activity of 1.4 % hemifusion and 0.04 FF/RL similar to the two negative controls, Mut9 (1.3 % hemifusion and 0.3 FF/RL) and Mut10 (1.2 % hemifusion and 0.01 FF/RL; Fig. 2, b).

We then tested the combination of HE0/F-Nev, the latter referring to the F protein of ISAV Nevis. This combination had a mean fusion-triggering activity of 15.9 % hemifusion and 2.60 FF/RL (Fig. 3a, b), in strong contrast to the low activity recorded for HE0/F-Gle (Fig. 2a, b). Three engineered HPR deletions, Mut4/F-Nev (HPR4), Mut6/F-Nev (HPR14) and Mut7/F-Nev (HPR9), demonstrated significantly increased fusion-inducing activity ranging from 37.6 to 60.1 % hemifusion and 4.16 to 10.96 FF/RL (Fig. 3a, b). In contrast, Mut2/F-Nev (HPR7), Mut3/F-Nev...
(HPR2) and Mut5/F-Nev (HPR8) produced hemifusion levels similar to HE0/F-Nev, ranging from 22.3 to 22.6 %, but lower content mixing levels than HE0/F-Nev, ranging from 0.77 to 1.38 FF/RL. Interestingly, and in contrast to the content mixing results using F-Gle (Fig. 2a, b), Mut8/F-Nev, which did not correspond to any known ‘natural’ HPR deletion, induced a significantly higher fusion level of 36.9 % hemifusion and 6.63 FF/RL (Fig. 3a, b). Overall, six of the seven HE deletions (Mut2–7) combined with F-Gle (Fig. 2a, b) and four of the seven (Mut4 and Mut6–8) combined with F-Nev (Fig. 3a, b) resulted in a significant increase in fusion-inducing activity compared with the HE0 reference protein. Taken together, these findings strongly suggested that the HPR deletions influence the fusion process and predominantly enhance the induction of fusion.

The position and length of the HPR deletions does not influence fusion activity, while the amino acid composition of the associated F protein does

After having shown that deletions in the HPR region influenced the fusion process, we analysed in more detail to

<table>
<thead>
<tr>
<th>Name</th>
<th>HPR type</th>
<th>Position</th>
<th>HPR sequence (aa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE 0</td>
<td>HPR0</td>
<td>N/A</td>
<td>TVKIRVDAIPQNLNQVPNQVEQPSVLSNIFISGMVA</td>
<td>McBeath et al. (2009)</td>
</tr>
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<td>Mut 2</td>
<td>HPR7</td>
<td>339–361</td>
<td>TVK---------------------------------------------------</td>
<td>Rimsd et al. (2001)</td>
</tr>
<tr>
<td>Mut 3</td>
<td>HPR2</td>
<td>352–371</td>
<td>TVKIRVDAIPQNLQT-------------------------------------</td>
<td>Rimsd et al. (2001)</td>
</tr>
<tr>
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<td>349–365</td>
<td>TVKIRVDAIPQ-------------------------------------------</td>
<td>Johnson et al. (2008)</td>
</tr>
<tr>
<td>Mut 6</td>
<td>HPR14</td>
<td>344–352</td>
<td>TVKIRVDA----------QVEQPSVLSNIFISGMVA</td>
<td>Kibenge et al. (2007)</td>
</tr>
<tr>
<td>Mut 8</td>
<td>N/A</td>
<td>339–371</td>
<td>TVK---------------------------------------------------</td>
<td>33 aa of HPR removed</td>
</tr>
<tr>
<td>Mut 9</td>
<td>N/A</td>
<td>307–361</td>
<td>-----------------------------------------------------</td>
<td>Heptad repeat removed</td>
</tr>
<tr>
<td>Mut 10</td>
<td>N/A</td>
<td>N/A</td>
<td>ISMKIRVDAIPQNLNQVPNQVEQPSVLSNIFISGMVA</td>
<td>Double HPR present</td>
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</table>

**Fig. 1.** Position and source of the various segment six HPR mutations. All mutated sequences were constructed from the ‘HE0’ template (row one). In the HPR sequence, the C-terminal region of the heptad repeats (boxed) and N-terminal of the transmembrane domain (underlined) demarcate the HPR boundaries. Mut2–7 deletions were based on those reported from pathogenic ISAV. Mut8 consisted of a 33 aa HPR deletion (not reported in the wild). Mut9 consisted of an HPR7 deletion with additional removal of predicted heptad repeat and Mut10 consisted of a full 35 aa HPR inserted in addition to the one already present.

**Fig. 2.** Fusion levels of mutant HE co-expressed with the F Glesvaer protein. Fusion activity was measured by lipid mixing (a) and a content mixing assay (b), which illustrated hemifusion and full fusion, respectively. The positive control (Glesvaer HE and F) is indicated by HE-Gle; Mut9 and 10 served as negative controls. Results are the means from two independent replicate experiments and are expressed as a percentage of the monolayer showing fusion adjusted to the average RL activity (a), and as the FF/RL ratio with FF expression correlating directly to the amount of cytoplasmic transfer (b). Vertical lines indicate SE.

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what extent the position and the length of the HPR deletion influenced fusion activity. We were unable to identify any clear pattern when comparing the position (Fig. 1) of the deletion with the lipid and content mixing assay data (Figs 2 and 3). This was best illustrated by Mut2 (aa 339–361) and Mut3 (aa 352–371), which carried divergent deletions with only a 10 aa similarity (Fig. 1), but were found to induce comparable fusion levels when combined with F-Nev at 22.5% hemifusion and 0.77 FF/RL, and 22.3% hemifusion and 1.28 FF/RL for Mut2 and 3, respectively. Similarly, when combined with F-Gle, the results were 46.3% hemifusion and 11.67 FF/RL, and 28.7% and 8.47 FF/RL for Mut2 and 3, respectively.

When we compared the length of the deletion, we also could not identify any clear pattern as to how this influenced fusion activity (Figs 1–3). This was exemplified by the most divergent HPR lengths, the original HE0/F-Gle and the HE Mut8/F-Gle (33 aa deletion) inducing relatively similar levels of fusion activity at 1% hemifusion and 0.12 FF/RL, and 1.4% hemifusion and 0.42 FF/RL for HE0 and Mut8, respectively. Finally we also found that Mut5 (HPR8), which is of particular interest since the amino acid near the proposed transmembrane domain (TMD), M372, is omitted in this mutant, systematically induced low levels of fusion activity in the content mixing assay, with 1.65 FF/RL for the F-Gle and 1.38 FF/RL for F-Nev protein (Figs 2b and 3b).

We were then left with the observation that the origin of the F protein had a stronger influence on fusion activity than particular deletions of the HE protein, most clearly shown when F-Gle and F-Nev were combined with the HE0 protein (Figs 2b and 3b), where there was an extremely low content mixing level for HE0/F-Gle (0.12 FF/RL), approximately 22 times lower than for HE0/F-Nev (2.60 FF/RL). Similarly, the HE Mut8/F-Gle (33 aa deletion) also induced a significantly lower content mixing activity of 0.04 FF/RL (Figs 2b and 3b), approximately 166 times lower than for Mut8/F-Nev (6.63 FF/RL). These findings indicated that some of the amino acid differences between the F protein of Glesvaer (HPR2) and the Nevis strain (HPR7) play a role in virus fusion in conjunction with the segment 6 HPR deletions. However, the importance of the different positions remains to be elucidated.

**HPR deletions influence the degree of dissociation between the F and HE proteins**

From the studies above, we had indications that a shortening of the HPR influences fusion and possibly the interaction between the HE and F proteins. To test this further, we used an assay where we stained HE and F proteins expressed on the surface of transfected salmonid cells and used confocal microscopy to measure their degree of co-localization. Such an approach is commonly used to analyse protein–protein interactions (Berggård et al., 2007). These HE/F-expressing cells were then subjected to haemadsorption with labelled EGs as ligands for the expressed HE protein (Fig. S2c). Following transfection, most co-expressed HE and F proteins were generally encountered in the same physical location and formed defined clusters of glycoproteins on the surface of the cells (Fig. 4a, b). A similar glycoprotein distribution has been observed on the surface of salmonid cells infected with ISAV (data not shown). In the monolayers not subjected to any haemadsorption with EGs, the five different HE proteins selected (HE0 and Mut2, 3, 8 and 9) and co-expressed F-Gle proteins were generally encountered in the same physical location and formed defined clusters of glycoproteins on the surface of the cells (Fig. 4a, b). A similar glycoprotein distribution has been observed on the surface of salmonid cells infected with ISAV (data not shown). In the monolayers not subjected to any haemadsorption with EGs, the five different HE proteins selected (HE0 and Mut2, 3, 8 and 9) and co-expressed F-Gle proteins systematically demonstrated a high percentage of co-localization, ranging from 79.2% for Mut8 to 85.5% for HE0 (Fig. 5a).

In the transfected cells subjected to EG haemadsorption (Fig. 5b), the HE and F protein co-localization was similarly high in negative areas of the cell surface (areas devoid of EG binding) ranging from 72% for Mut9 to 83% for Mut3. The most interesting findings came from positive areas (on the same cell) where HE proteins were engaged in binding of EGs and the percentage of HE and F...
protein co-localization was systematically lower, ranging only from 42% for Mut2 to 63% for Mut9 (Fig. 5b). HE0 and Mut9 demonstrated the lowest level of dissociation at 14 and 9% respectively (Fig. 5b). These were also the HE variants that gave some of the lowest degrees of fusion in the lipid and content mixing assay when combined with F-Gle (Fig. 2a, b). In contrast, Mut2, 3 and 8, which harboured an HPR deletion, all displayed significantly higher levels of surface proteins dissociation compared with HE0, ranging from 32 to 36% (Fig. 5b). Again, this is concordant with higher fusion levels recorded for Mut2 and 3 when combined with F-Gle but not with Mut8 (Fig. 2a, b). From these studies, we concluded that HE and F are tightly co-localized on the surface of salmonid cells, and that HPR deletions in HE may facilitate dissociation between the F and HE proteins following receptor binding.

**DISCUSSION**

This study represents the first functional analysis of the HE protein HPR and its implication in the induction of ISAV fusion. ISAV has a similar functional organization of its attachment and fusion surface proteins to many paramyxoviruses (Lamb & Kolakofsky, 2001; Li et al., 2004; Tanabayashi & Comans, 1996; Tsurudome et al., 1995). Like these viruses, ISAV surface glycoproteins act

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**Fig. 4.** Antibody co-patching and confocal microscopy photos (×40) for the HE Mut3 co-expressed with the F Glesvaer protein. HEs were labelled in green (Alexa Fluor 488) and the F proteins in red (Alexa Fluor 594) and appear as yellow aggregates when highly co-localized. Some cell monolayers were left untreated (a), while others were subjected to haemadsorption with DAPI-labelled EGs with blue nuclei (b).

**Fig. 5.** Box plot showing percentages of co-localization between different HE and co-expressed F Glesvaer proteins at the surface of transfected cells. Co-localization was measured after 48 h incubation with no treatment (a) and after EG haemadsorption (b). In (b), the percentages listed above indicate the difference between the negative areas (−, no EGs binding) and positive areas (+, halo of binding around the EGs), which corresponds to the level of dissociation between the two surface proteins. Data are displayed as the mean (horizontal line) of at least 20 or more binding events, with an additional indicator of 50% data distribution (box) ± SE. Data outliers are indicated by ○. *0.01 < P ≤ 0.05; ***P ≤ 0.001.
cooperatively to induce fusion, with the HE protein physically interacting with and most likely activating the F protein. More specifically, the membrane proximal HPR of the HE stalk and associated deletions were found to influence ISAV fusion. In paramyxoviruses, residues in the H or haemagglutinin–neuraminidase protein near the membrane stalk domain have been identified as important sites interacting with the F protein and are critical to the fusion process (Brindley & Plumper, 2010; Lee et al., 2008; Melanson & Iorio, 2004; Zhou et al., 1997).

In most cases, deletions generated in a ‘full-length’ HPR resulted in a mutant HE with an increased fusion-inducing ability compared with the original undeleted HPR0 protein. Therefore, a shortening of the HPR appears to influence the interaction with the F protein in a way that promotes the induction of viral fusion. One study on measles virus investigated the effect of deletions within the membrane proximal region of the H stalk by removing heptad repeat elements spanning six residues (Paal et al., 2009). These relatively small deletions, compared with those observed in ISAV, had a deleterious effect on fusion activity, because they altered the location of residues acting as essential points of contact for the docking of the F protein globular head (Paal et al., 2009). A heptad repeat was predicted in ISAV segment 6 (Fig. S1) using the web-based predictor programs Matcher and LearnCoil-VMF (http://cis.poly.edu/~jps/matcher.html; http://groups.csail.mit.edu/ch/learncoil/vmf/cgi-bin/vmf.cgi), which corresponded to the part of the HE stalk located immediately upstream of the HPR. The removal of the entire predicted heptad repeat sequence (Mut9) was found to systematically eliminate the fusion-inducing ability of the HE protein, suggesting the presence of an essential F-interactive domain as described for paramyxoviruses (Gravel & Morrison, 2003; Melanson & Iorio, 2004; Wang et al., 2005). However, different lengths of HPR deletions downstream of the heptad repeat did not affect ISAV fusion as it did for measles virus (Paal et al., 2009) and actually promoted, in some cases significantly, the fusion-inducing ability of the attachment protein. Even an artificial and substantial deletion of 33 aa (Mut8) still induced a high level of fusion when combined with F-Nev.

Previous research has proposed that the length of deletion and presence of certain HPR motifs are important determinants of ISAV virulence. Deletions of 13 aa or less and the presence of a specific ‘FNT’ motif at positions 352–354 were correlated with a reduced ability to produce any cytopathic effect in cell culture or any disease in Atlantic salmon (Kibenge et al., 2007). The amino acid sequence NIT at positions 333–335 upstream of the HPR, and NQT at positions 349–351, were reported as important putative glycosylation sites (Mikalsen et al., 2005; Mjaaland et al., 2002), the latter being associated with reduced ISAV virulence (Kibenge et al., 2004). The present findings indicate that the position and length of HPR deletions are not critical factors in the context of fusion induction. The presence of NQT and FNT in Mut7 or their complete absence in Mut6 resulted in varied levels of fusion induction depending on the co-expressed F protein. The NIT glycosylation site was located in the predicted heptad repeat and was removed as part of the Mut9 deletion, which was systematically associated with extremely low fusion activity. However, since the Mut9 deletion was extensive (55 aa omitting both heptad repeat and HPR), it would be difficult to attribute this effect specifically to the absence of the NIT motif. The lack of a specific effect from any particular section of the HPR may indicate that the mechanisms by which HPR deletions influence fusion induction may be quite generic and operate in a non-specific fashion.

Only the Mut5 HE protein was consistently associated with a low level of content mixing, but a moderate percentage of hemifusion (Figs 2 and 3). This HPR8 deletion (Fig. 1) was the only one omitting the methionine at position 372, which is located at the boundary between the HPR and predicted N-terminal part of the TMD (Nylund et al., 2003) or within the TMD itself (Kibenge et al., 2007). In influenza viruses, the TMD plays an important role during the transition from hemifusion to the full fusion state (Chang et al., 2008; Kemble et al., 1994; Markosyan et al., 2000; Melikyan et al., 1999), and deletions in this region can drastically impede content mixing (Armstrong et al., 2000). Moreover, among pathogenic ISAV isolates, HPR deletions encompassing the methionine at position 372 are scarce (Kibenge et al., 2007; Mjaaland et al., 2002; Nylund et al., 2003), which may suggest that this particular residue is functionally important.

In comparison, the segment 5 amino acid composition was a more crucial factor influencing viral fusion in conjunction with segment 6 HPR deletions. The F proteins of the pathogenic Nevis (HE HPR7) and Glesvaer (HE HPR2) strains differ by eight residues (data not shown) and some of these had a profound effect on the ability of the different HE proteins to induce fusion. This was particularly striking with the HE0 protein, which displayed very poor fusion-inducing ability with the F-Gle protein, while inducing moderate levels with F-Nev. This illustrated that, despite a ‘full-length’ HPR sequence, the HE0 protein was still capable of full fusion and delivering the FF reporter gene in the target cell, depending on its associated F protein.

Antibody co-patching and co-localization analysis provided several important new findings regarding the ISAV fusion strategy. First, in the monolayers not subjected to any haemadsorption, the HE and F proteins were highly co-localized forming defined clusters of glycoproteins dotted over the entire cell surface (Fig. 4a, b). Such close proximity suggested that the two surface proteins must interact physically with each other (Berggård et al., 2007). Secondly, the presence of these glycoproteins aggregates immediately after transfection and following 48 h incubation suggested that the HE and F proteins may already interact with each other as they reach the cell surface. Thirdly, haemadsorption of EGs significantly decreased the
percentage of HE and F protein co-localization in the area where receptor engagement was predicted. This suggested that receptor binding promoted physical separation between the HE and F proteins. All together, these results suggested that ISAV may be following a ‘dissociation model’ akin to that proposed for measles virus (Plemper et al., 2011; White et al., 2008) where the release of the F protein from the H protein activates the fusion process (Brindley et al., 2012; Paal et al., 2009; Plemper et al., 2001). However, further work would be required to confirm this theory.

Interestingly, all of the HPR deletions selected (Mut2, 3 and 8) significantly increased the level of F-Gle protein dissociation following receptor binding, while the full-length HPR HE0 only generated a moderate degree of release. Previous work has reported the importance of the strength of interaction between the attachment and F protein in regulating fusion activation and viral cytopathogenicity (Aguilar et al., 2006, 2007; Plemper et al., 2002). In ISAV, the HE stalk length may be an important factor influencing the avidity between the two surface glycoproteins, with HPR deletions potentially reducing the strength of interaction and the energy requirement to release the F protein as reported with other viruses (Ayllón et al., 2010; Plemper et al., 2002). The percentage of dissociation observed in Fig. 5 correlated well with the fusion activity recorded in Figs 2 and 3 with the exception of Mut8, which demonstrated a high release of F protein but a poor fusion-inducing capacity with the F-Gle protein. This last result indicated that other fusion mechanisms, such as the refolding of the F protein into a metastable conformation (Ayllón et al., 2010) and the proteolytic cleavage (Aspehaug et al., 2005) might also play an important role in promoting the final fusion step.

To conclude, this study identified that the segment 6 near-membrane HPR plays an important role in ISAV fusion. Deletion within the HPR predominantly enhanced the fusion-inducing ability of the resulting mutant HE. We propose that, for ISAV, receptor binding onto the HE protein promotes the physical separation of the F protein and potential activation of viral fusion. In this process, HPR deletions appear to reduce the strength of interaction between the two glycoproteins, and inversely enhance the F protein dissociation. However, further work would be required to confirm this theory. The position and length of the HPR deletions were not important factors, suggesting a non-specific effect. In comparison, the segment 5 amino acid composition was a more important criterion influencing viral fusion in conjunction with segment 6 HPR deletions. Finally, these results may provide a first insight into the mechanism of virulence acquisition through HPR deletion, since newly formed virions inheriting shorter-stalked HE proteins could be more efficient at delivering their genomic material into host cells.

METHODS
Recombinant plasmids and mutagenesis. Details of the construction of the plasmids expressing the segment 6 HPR0 NWM10, Nevis 390/98 (Fig. 1) and Mut2 have been described elsewhere (McBeath et al., 2011). The segment 5 (F) of Nevis 390/98 (GenBank accession no. AJ277461) and Glesvaer (GenBank accession no. AF429987), and segment 6 of Glesvaer (GenBank accession no. AF220607; Fig. 1), were produced similarly. These plasmids were used as templates for subcloning into phrGFP-1a (Stratagene). Briefly, inserts were excised by SacI and Xhol digestion (New England Biolabs) and ligated in the phrGFP-1 plasmid, thereby replacing the hrGFP portion. All products were ligated and purified as described previously (McBeath et al., 2011).

The additional mutants, designated Mut3–10, were designed using the full-length HPR0 NWM10 with the removal of HPR sections based on deletions reported in several pathogenic viruses (Fig. 1). Mut8, with 33 aa removed, did not correspond to a naturally occurring mutation. Drastic structural changes were incorporated into Mut9 and Mut10 in order to eliminate fusion activity and create negative controls. Constructs were synthesized by Genecust, propagated and subcloned as described previously (McBeath et al., 2011).

Cell culture and transient transfection system. Chinook salmon embryo (CHSE-214; ATCC 1681) cells were cultured, and transfected as described previously (McBeath et al., 2011) using a Neon 10 μl kit (Invitrogen), and a total of 2.5 μg DNA per reaction. Prior to this study, a F/HE plasmid ratio of 3/1 was identified as the optimal combination of surface proteins (data not shown), corresponding to 1.5 and 0.5 μg, respectively. Each reaction was also co-transfected with 0.5 μg plasmid expressing the RL gene with a cytomegalovirus (CMV) promoter (Promega) to correct for variations in transfection efficiency. Reactions were subjected to electroporation conditions of two 20 ms pulses of 1300 V and added to 3 ml culture medium. This cell solution was dispensed into culture plates and the monolayers were incubated for 48 h at 20 °C.

Lipid mixing assay. A total of four 96-well plates were prepared for each group, three undergoing treatments and one remaining untreated. Fresh Atlantic salmon red blood cells (RBCs) were labelled with the lipid probe R18 (Sigma, as described previously (Hoekstra et al., 1984). Haemadsorption, trypsin and low-pH treatment were applied as described previously (Aspehaug et al., 2005) using 0.075 % R18-labelled RBCs and a solution of 2.5 mg trypsin ml⁻¹. Monolayers were observed under a digital inverted fluorescent microscope (EVOS, AMG) at ×10 magnification under red fluorescence conditions (531 nm excitation/593 nm emission). Five non-overlapping fields of view (approx. 15 % of the well surface) were photographed in set areas. These were selected to include a fully confluent cell monolayer and display comparable levels of bound erythrocytes and therefore HE surface expression. Transfection efficiency was estimated by measure of RL activity by luminometry using a Dual-Glo Luciferase Assay System (Promega) and a Viktor Multireader (PerkinElmer) over 10 s. Image analysis of fusion events was later performed using image analysis software (Cell Olympus Soft Imaging Solution, version 2.4.206-141206). Results were expressed as the percentage of the monolayer showing fusion and corrected for transfection efficiency using the average RL activity. A two-way ANOVA was performed on the logged fusion data, using the statistical R package (http://www.R-project.org) and adjusted for multiple comparisons with a Bonferroni correction.

Content mixing assay. A total of six 48-well plates were cultured for each combination of HE and F proteins on two separate plates, one undergoing fusion treatments and one remaining untreated. One hundred microlitres of fresh Atlantic salmon blood was centrifuged once to remove the serum and the RBC pellet rinsed three times in Dulbecco’s PBS. Fifteen microlitres of the clean RBC pellet was mixed with 50 μl buffer T (Neon 10 μl kit; Invitrogen) and 10 μg plasmid expressing FF with a CMV promoter (Collet & Secombes, 2005). Five
10 μl reactions of this solution were then subjected separately to electroporation conditions of one 20 ms pulse of 1900 V and pooled together into 25 ml L-15 medium. This stock solution of EGs carrying plasmids was then used for haemadsorption, and one plate was subjected to trypsin and low-pH steps as described previously (Aspehaug et al., 2005), while the other remained untreated. The two plates were then incubated for an additional 48 h at 20 °C. Both FF and RL protein expression was measured with a Dual-Glo Luciferase Assay System (Promega). Content mixing results were expressed as FF/RL ratio and values from the untreated plate were subtracted from the final measurements. Statistical analysis was performed as described above.

Immunofluorescence co-patching. Five combinations of HE and F-Cie plasmids were transfected into CHSE-214 cells and cultured onto eight-well chamber glass slides (BD Falcon). RBC pellets were prepared as described above and the fluorescent label DAPI (Invitrogen) was loaded into the erythrocytes by osmotic lysis of pinocytic vesicles as described previously (Okada & Rechsteiner, 1982) to ensure visualization of binding. The first well of transfected cells was left untreated while another three were subjected to haemadsorption with 3% DAPI-labelled EG solution as described above.

The co-localization of HE and F proteins at the surface of transfected cells was measured using antibody co-patching and confocal microscopy as described previously (Keren et al., 2001; Shvartsman et al., 2003). All further incubations and steps were performed on living cells to ensure exclusive surface membrane labelling by the antibodies, and at 4 °C, in the dark, with pre-chilled L-15 to minimize cellular internalization. Briefly, both the untreated and EG-treated cell monolayers were incubated with 1% non-fat dried milk solution (Sigma) in L-15 for 1 h to block non-specific binding. This was followed by incubation with a rabbit polyclonal antibody targeted at ISAV F protein (anti-EB5Bac; Aspehaug et al., 2005) diluted in a 0.5% non-fat dried milk solution in L-15 (1:450) for 2 h and a cocktail of mouse mAb targeted at ISAV HE protein (3H6F8 and 10C9F5; Falk et al., 1998) diluted in the same non-fat dried milk solution (1:60) for 1 h. Following two rinses in L-15, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h (1:600). Finally, the monolayers were fixed as described previously (Gilboa et al., 2000), mounted with Prolong Gold Antifade (Invitrogen) and analysed using an LSM 710 confocal microscope (Zeiss). Surface expression of the different glycoproteins was also monitored and found to be comparable between each group (data not shown). The coefficient of co-localization of green (HE protein) and red (F protein) pixels on the surface of transfected cells was measured using the ZEN 2009 image analysis software (Zeiss).

The untreated monolayers were used to assess the co-localization status of HE and F proteins directly after transfection and prior to receptor engagement in binding. In this situation, measurements were recorded from the entire surface area of 20 cells per group. In comparison, haemadsorption of DAPI-labelled EGs allowed identification of the area of the cell surface engaged in receptor binding and evaluation of the effect on HE and F protein co-localization. As in previous observations (Hotchin et al., 1958), attached EGs were slightly deformed and embedded inside the lipid membrane of the transfected cells. This implied that, in this area around the edge of an embedded EG, HE proteins engaged in binding would still be accessible for antibodies and visualization (Fig. S2c). HE and F protein co-localization coefficients were therefore measured in this 'halo of binding' around the edge of the erythrocyte and designated a positive area. These were directly compared with the co-localization coefficient on the same cell surface, in a negative area without any binding. A minimum of 20 or more binding events and their associated negative areas were measured independently and compared for each group. A linear mixed modelling approach was used to analyse the logged co-localization data using the statistical R package.

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