Characterization of the Infectious Salmon Anemia Virus Fusion Protein

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Infectious salmon anemia virus (ISAV) is an orthomyxovirus causing serious disease in Atlantic salmon (*Salmo salar* L.). This study presents the characterization of the ISAV 50-kDa glycoprotein encoded by segment 5, here termed the viral membrane fusion protein (F). This is the first description of a separate orthomyxovirus F protein, and to our knowledge, the first pH-dependent separate viral F protein described. The ISAV F protein is synthesized as a precursor protein, F₀, that is proteolytically cleaved to F₁ and F₂, which are held together by disulfide bridges. The cleaved protein is in a metastable, fusion-activated state that can be triggered by low pH, high temperature, or a high concentration of urea. Cell-cell fusion can be initiated by treatment with trypsin and low pH of ISAV-infected cells and of transfected cells expressing F, although the coexpression of ISAV HE significantly improves fusion. Fusion is initiated at pH 5.4 to 5.6, and the fusion process is coincident with the trimerization of the F protein, or most likely a stabilization of the trimer, suggesting that it represents the formation of the fusogenic structure. Exposure to trypsin and a low pH prior to infection inactivated the virus, demonstrating the nonreversibility of this conformational change. Sequence analyses identified a potential coiled coil and a fusion peptide. Size estimates of F₁ and F₂ and the localization of the putative fusion peptide and theoretical trypsin cleavage sites suggest that the proteolytic cleavage site is after residue K²⁷⁶ in the protein sequence.

Infectious salmon anemia virus (ISAV) is an enveloped virus belonging to the family *Orthomyxoviridae* and the genus *Isavirus*, and it causes serious disease in Atlantic salmon (*Salmo salar* L.) (51, 58, 68, 71, 80). The ISAV genome is composed of eight negative-sense, single-stranded RNA segments, and while nucleotide sequences of all segments have been published (9, 43, 44, 56, 66, 67, 74, 75), much remains to be elucidated with respect to protein identification and characterization. A total of 12 proteins have been detected by immunoprecipitation of lysates from radiolabeled infected cells (40), while four major structural proteins have been recognized in purified ISAV particles, including the matrix (M1; 22 to 24 kDa) (7, 24), the nucleoprotein (NP; 66 to 71 kDa) (3, 24), and two membrane glycoproteins (24). The receptor-binding and receptor-destroying activities are associated with the 42-kDa glycoprotein encoded by segment 6, termed the hemagglutinin-esterase (HE) (24, 25, 34, 42, 43, 66), while the activities of the second glycoprotein, glycoprotein 50 (gp50), encoded by segment 5 (9), have not been described previously.

ISAV pursues a nuclear replication strategy similar to that of the influenza viruses (3, 26) which is initiated by receptor binding and internalization into cellular endosomes, where the viral and cellular membranes fuse in response to low pH (21). The addition of trypsin to the culture medium during ISAV replication has been demonstrated to have a beneficial effect

on the production of infective virus particles (26), suggesting that ISAV replication depends on proteolytic activation to exert infectivity.

Fusion between viral and cellular membranes is mediated by viral membrane fusion glycoproteins, which are usually divided into two distinct categories. Type I fusion proteins are activated by proteolytic cleavage and include the fusion proteins of orthomyxo-, paramyxo-, retro-, and filoviruses (19). Type II fusion proteins comprise the fusion proteins of flaviviruses, alphaviruses, and most probably the bunyaviruses, and these do not depend on proteolytic cleavage for activation but are associated with a second protein whose cleavage is essential for establishing fusion competence (28, 32, 33).

Type I fusion proteins are synthesized as single-chain precursors which assemble into homotrimers (13). An essential step in rendering the proteins fusion competent is the cleavage of the protein by host proteases to generate two subunits bound together by disulfide bridges (48). This cleaved form of the protein is in a metastable state, trapped from achieving its lower energy state by a kinetic barrier, and is readily activated by destabilization caused by events such as receptor binding or low pH (48). Once activated, the protein refolds into a highly stable conformation (12). Type I fusion proteins contain a hydrophobic sequence, known as the fusion peptide (FP), at or near the new amino-terminal region created by the cleavage event (57, 62, 76), and their final (postfusion) state contains a characteristic α -helical coiled-coil core structure corresponding to a heptad repeat motif (12, 57). In recent years, these structures have been targets for the development of new antiviral drugs, including fusion-inhibiting peptides and antibodies

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(20, 50, 82), suggesting that fusion proteins might be important candidates both for vaccination and as therapeutic targets.

The fusion of influenza A viruses to the cell membrane is mediated through hemagglutinin (HA), and although the pathogenicity of influenza viruses is polygenic, numerous studies have established that the cleavage-site structure of the HA precursor is the most significant determinant of pathogenicity (reviewed in references 64 and 77). Generally, HAs of avirulent strains are cleaved by a restricted number of host proteases. More virulent strains convey enhanced HA cleavability due to basic residues within the cleavage site and can be cleaved by a wide range of host proteases, leading to systemic infection and a higher pathogenicity. A similar correlation between F protein cleavage-site structure and pathogenicity has been observed in paramyxoviruses (30, 78, 81, 85) and may also be important in the pathogenicity of ISAV. Thus, the identification of the F protein and its proteolytic cleavage site might be key factors in establishing the virulence factors of ISAV.

The present study was undertaken to verify that ISAV genomic segment 5 encodes the 50-kDa glycoprotein and to demonstrate that it is the viral F protein. We have shown experimentally that the encoded protein exhibits the general characteristics described for type I viral fusion proteins.

MATERIALS AND METHODS

Viruses and cells. The ISAV isolates used in the experiments were strains Bremnes/98 (45) and Glesvaer/2/90 (15), as indicated. The virus isolates were propagated in salmon head kidney (SHK-1) cells as previously described (14). Functional assays were performed using SHK-1 cells, Atlantic salmon kidney (ASK) cells (18), and Chinook salmon embryo (CHSE-214) cells (49). Infections were performed as described by Falk et al. (26).

Sf9 cells were grown in suspension culture, infected with baculovirus at 28°C, and harvested at 5 days postinfection (p.i.) by centrifugation at 750 × g for 20 min at 4°C. The cell pellet was resuspended in 10 mM phosphate-buffered saline (PBS; pH 7.4) with 0.2% Triton X-100 (Amersham Biosciences) and then used for immunization.

Purified ISAV preparations were prepared by sucrose gradient centrifugation using the Bremnes/98 isolate, and infectivity titrations of ISAV and virus neutralization assays were performed by end-point titration in 96-well culture plates, using an immunofluorescence assay, all as described previously (25, 26).

Cloning and sequencing. A unidirectional cDNA library from ASK cells infected with the ISAV Bremnes/98 isolate was constructed in bacteriophage lambda, as previously described (43). A clone, designated EB5, was identified by immunoscreening with a polyclonal anti-ISAV rabbit serum (ISAV-PAb) (see below) using a picoBlue immunoscreening kit (Stratagene). The EB5 cDNA insert was excised as described previously (43) and then sequenced. The full-length cDNA sequence was obtained by 5' rapid amplification of cDNA ends (5' RACE system version 2.0; Invitrogen). RACE products were cloned in the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) and then sequenced.

To amplify the coding region of the EB5 cDNA insert, the primers EB5-EcoRI (5'-GAGCTAACAGAGAGGAATTCATGGCTTTTCTAAC-3') and EB5-XhoI (5'-CCAGCAGAACTCGAGTTATCTCCTAATGC-3') were used. The PCR product was subsequently cloned into pFastBac1 and pVAX1 vectors (Invitrogen), using the EcoRI and XhoI restriction enzymes (Promega). These vectors were named pFastBac1/EB5 and pVAX1/EB5, respectively.

For the expression of ISAV HE in fish cells, the vector pVAX1/HE was constructed. The ISAV Bremnes/98 HE gene (43) was amplified by PCR using the primers HE-KpnI-f (5'-GGGTACCGCAAAGATGGCACGATTC-3') and HE-XhoI-r (5'-CCGCTCGAGGTTGCTTTCTTTCATAATC-3') and then cloned into the KpnI and XhoI restriction sites of the pVAX1 vector.

The pFastBac1/EB5, pVAX1/EB5, and pVAX1/HE plasmids were all used in transformations of *Escherichia coli* TOP10 cells (Invitrogen), purified using an endotoxin-free plasmid Midi kit (QIAGEN), and sequenced using a BigDye Terminator sequencing kit (PE Biosystems) and an ABI 377 DNA analyzer (PE

Biosystems). Sequences were assembled and analyzed as previously described (43).

Computer-based protein sequence analyses. Sequence database searches were performed using BLAST (1). Pairwise sequence alignments were performed using ClustalX (79) and viewed using GeneDoc (59). Primary and secondary structure predictions were performed using the ExPASy tools, including Compute pI/MW and PeptideCutter (29). DISULFIND was used to predict disulfide bridges (84). Posttranslational modifications were predicted using SignalP (5), NetOglyc 2.0 (31), and Motif Scan (60), while topology predictions were done by TopPred2 (83), SPLIT (39), and TMPred (36). Learncoil-VMF, a program specialized for the prediction of coiled-coil domains of viral membrane F proteins (73), was used to search for sequences with the probability of forming α -helical coiled coils, using a probability of 0.5 as the cutoff value. The program COILS, which estimates the probability that a sequence will adopt a coiled-coil conformation, was also used (52). Structure homology searches were done by FUGUE (72) and PDB-BLAST (2). Tertiary structure predictions based on structural homology were done by TIT0/MODELLER 4.0 (27, 53, 70), using a PDB structure of the visna virus Env coiled coil (1jek) to perform three-dimensional (3D) modeling.

Expression of EB5 cDNA in Sf9 cells. Purified pFastBac1/EB5 was used to transform *E. coli* DH10Bac competent cells (Invitrogen). Recombinant bacmid was obtained from the transformed DH10Bac cells and used to recover recombinant baculovirus (BacEB5) after transfection of Sf9 cells according to the method described for Bac-to-Bac kits (Invitrogen). BacEB5 was used to express the EB5 protein in Sf9 cells according to the recommendations of the manufacturer. The expression level and specificity of the recombinant EB5 protein expressed in Sf9 cells were verified using the ISAV-PAb (see below) for immunoblotting and immunostaining of EB5-expressing cells (results not shown).

Antisera. An antiserum against two synthetic peptides derived from the EB5 sequence was prepared by Eurogentec using the peptides HWTTSRSRLDSTWQGG-NH₂ and HFTTERIKTGKVDLDSC-NH₂ (Fig. 1) coupled to keyhole limpet hemocyanin. The conjugated peptides were pooled and injected into a rabbit according to Eurogentec's procedures, resulting in the antiserum termed anti-pepEB5.

A synthetic peptide (GAASAEDVKEKLNIGIDQINKVNLLEGEIEAVRR IAYMN-NH₂) corresponding to the predicted EB5 coiled-coil sequence (Fig. 1; see Results) was synthesized by Sigma-Genosys Ltd., purified to >95% purity by high-performance liquid chromatography, and coupled to keyhole limpet hemocyanin. The conjugated peptide was injected into a rabbit according to Sigma-Genosys Ltd.'s procedures, resulting in the antiserum named anti-pepcoilEB5.

An antiserum against the baculovirus-expressed EB5 protein (anti-EB5Bac) was prepared by immunizing a rabbit four times at 4-week intervals, using approximately 150 μ g protein from a crude lysate of Sf9 cells infected with BacEB5.

A polyclonal antiserum prepared against whole ISAV (ISAV-PAb) (43), a monoclonal antiserum directed against ISAV NP (anti-NP MAb; Aquaculture Diagnostics Limited), and a monoclonal antiserum directed against ISAV HE (anti-HE MAb; 3H6F8) (25) were also used in this study.

Transfections. Transfections were done on CHSE-214 cells grown on coverslips in 24-well plates using the FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's recommendations.

Also, for fusion assays, single and combined transfections of SHK-1 cells were performed with plasmid pVAX1/EB5 and/or pVAX1/HE using the Amara Nucleofector technology (Amara GmbH). A procedure optimized according to the manufacturer's protocol for the fish cell line TO by E. García-Rosado and coworkers (personal communication) was used. The pVAX1/EB5 and pVAX1/HE plasmids were used in 1:2, 1:1, and 2:1 ratios to transfect 5×10^6 cells, using a total of 9 μ g DNA for transfections using both plasmids and 7.5 μ g DNA for single transfections. The transfected cells were transferred into two parallel wells in a six-well plate and incubated as recommended for SHK-1 cells.

Hemadsorption and fusion assays. Hemadsorption for receptor binding studies and the lipid mixing fusion assay was performed using Atlantic salmon (*S. salar* L.) erythrocytes as previously described (43, 54). Two fusion assays were established using SHK-1 cells infected with the ISAV Glesvaer/2/90 isolate, namely, a lipid mixing assay based on the fusion between ISAV-infected cells and octadecyl rhodamine B (R18) (Molecular Probes) membrane-labeled Atlantic salmon erythrocytes (4) and a syncytium assay based on cell-cell fusion. For both assays, the infected cells were treated with trypsin (0, 10, 20, and 40 μ g/ml and 0, 2.5, 5, 10, 20, 40, and 60 μ g/ml, respectively) (Sigma) in Leibovitz L-15 medium (L-15; Gibco BRL) for 15 min at room temperature (RT). Trypsin was then inactivated by 10% fetal calf serum in L-15. For the lipid mixing assay, hemadsorption was performed using Atlantic salmon (*S. salar* L.) erythrocytes labeled with R18 as described previously (4). Cells in both assays were further incubated

Norway	:	MAFLITLVLFL LKEVLCEPC	TCDNPTCLGL TIPRTGYVRS	APGGVLLTET	ITESPALAEW	TSRSRLDS	TWQGEVKS	80
Canada	:	F.....	I.E.....I	QA.F.....	..R.Q.T.....	PK..ET	L.LD..T.N..	80
Norway	:	KVSQTLFEAI	QGTOMENCAV	KAVFDTSEVN	LTHHDVVLGR	VKVSPPGGEH	DISKCGRKL	160
Canada	:	K.....	L.T.....	KQ.I.....	K.I.....	E...DS	V.....	160
Norway	:	CRGRKGRMMS	LEPTADCGVE	KQPTTERIKT	GRVLDLSCCT	QHGCTKGIRV	EVPSPLVLSA	240
Canada	:	..K.K.....	..A.....T.....	..L.D.....	..ML.IT.....	S.....	..Q.VT.....	240
Norway	:	FARTSSFTLR	ANLANQHWS	KYSFNLRAFP	GEEPIKCCGF	TLGIGGAWFO	AYLNGEVQGD	320
Canada	:	K.....	FV.K.....	N.....	G.....	V.....	M.....	320
Norway	:	KVNLLLEGEI	EAVRIAYMN	QASSLQNVQE	IGLIGEYLN	SSWLETKILT	KTEEGLMKDG	400
Canada	:	..DT.....			T.....	N.....	..Q.NT.....	400
Norway	:	GYVDNIKEVT	GTSWVMIMH	YIIVGLIVVV	LVLGLKLMG	CIRR		444
Canada	:	S.....	I.....	V.F.....	L.....			444

FIG. 1. Pairwise sequence alignment of the genomic segment 5-encoded proteins of the Norwegian ISAV isolate Bremnes/98 (Norway) (accession no. CAD99169) and the Canadian isolate ME/01 (Canada) (accession no. AAN57720) (9). Predicted transmembrane α -helix regions are framed with boxes (residues M¹ to C¹⁷ [putative signal peptide], residues I²⁷⁵ to Y²⁹² [internal transmembrane region], and residues V⁴¹⁷ to W⁴³⁹ [putative C-terminal anchor]). Potentially O-glycosylated threonines T³² and T⁶² and N-glycosylation sites are underlined with plain and dashed lines, respectively, and are shown in bold. The putative fusion peptide region C²⁷⁷ to Y²⁹² is shown in italics. The putative coiled-coil domain (residues G³⁰¹ to N³⁴⁰) is underlined. Potential trypsin cleavage sites are shown with gray shading, while the ones at positions R²⁶⁷ and K²⁷⁶, considered the most likely proteolytic cleavage sites, are shaded in black. Cysteine residues putatively supporting disulfide bridges are shown in bold. The regions corresponding to the synthetic peptides used for immunizations to make the anti-pepEB5 antibody are shown with dashed boxes.

in L-15 at pH 3.5 to 8.5 for 15 to 30 min. Cells were washed in L-15 between all incubations. The results of the lipid mixing assay were read instantly by microscopy. The cells in the syncytium assay were further incubated in L-15 at RT for 2 h and kept at 4°C overnight prior to fixation and immunostaining as described below. After initial experiments, the optimized syncytium assay, using 20 μ g/ml trypsin and pH 4.5, was set as the standard fusion assay and used in repetitions with ASK cells and studies of transfected fish cells.

Immunofluorescence. Cells attached to coverslips were fixed with cold 3.7% formaldehyde in PBS for 10 min on ice, rinsed in PBS, and permeabilized with 0.2% Triton X-100 (Amersham Biosciences) in PBS for 30 min at RT, followed by blocking in 5% nonfat dry milk (Sigma) in PBS for 30 min. The cells were incubated with primary antibodies diluted in 2.5% nonfat dry milk in PBS for 1 h and washed in PBS. Bound antibodies were detected using Alexa dye 488-labeled anti-rabbit immunoglobulin G (IgG; Molecular Probes) and/or Alexa dye 594-labeled anti-mouse IgG (Molecular Probes). After being washed, the cells were mounted with SlowFade antifade medium (Molecular Probes) or Vectashield antifade medium containing DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories) and examined under a confocal microscope (TCS SP2 AOBIS; Leica) or a standard inverted microscope (Olympus IX81 or Nikon Eclipse TE 2000-E). Polyclonal antisera (including secondary antisera) were adsorbed by incubation on monolayers of formaldehyde-fixed and permeabilized ASK cells prior to use in immunofluorescence.

SDS-PAGE and Western blot (WB) analysis. Samples were treated with dissociation buffer (50 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], 50 mM dithiothreitol, 8 mM EDTA, 0.01% bromophenol blue) and heated for 5 min at 95°C. In nonreduced samples, dithiothreitol was omitted and samples were incubated for 30 min at RT. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous system devised by Laemmli (46), using 0.5-mm-thick precast 12.5% or 8 to 18% gradient polyacrylamide gels (ExcelGel SDS; Amersham Biosciences), and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences) as previously described (43). The membrane was treated with blocking solution (PBS, 0.1% Tween 20, 5% nonfat dry milk) overnight at 4°C, followed by a 1-h incubation at RT with the primary rabbit immune serum. The membrane was then washed three times in blocking solution and incubated with goat anti-rabbit horseradish peroxidase conjugate (AMDEX; Amersham Biosciences) for 1 h at RT. Bound horseradish peroxidase conjugate was detected using ECL Plus (Amersham Biosciences) and exposure on Hyperfilm ECL (Amersham Biosciences). The blots were scanned in a desktop scanner (CanoScan 3000F; Canon) and analyzed using Quantity One 1D gel analysis software (version 4.5.2; Bio-Rad).

A 2D electrophoresis was performed by using nonreducing SDS-PAGE for the first dimension. A lane excised from the polyacrylamide gel was immersed in dissociation buffer and heated for 5 min at 95°C before it was run in the second dimension by reducing SDS-PAGE and blotted as described above.

Trypsin digestion assay and gp50 homotrimer formation/stabilization. Samples were incubated with trypsin (Sigma) to a final concentration of 20 μ g/ml in PBS for 10 min at 37°C. The digestion was stopped by the addition of trypsin inhibitor from bovine pancreas (type I-P; Sigma) to a final concentration of 20

μ g/ml. After digestion, the pH of the samples was adjusted by diluting the samples 1:1 in 0.2 M citrate buffer (pH in the range of 4.5 to 7.3 $\pm$ 0.1), or the samples were exposed to 60°C or 4.5 M urea (final concentration), incubated for 1 h at RT, and analyzed under nonreducing conditions by SDS-PAGE and WB as described above.

Chemical cross-linking. Chemical cross-linking of purified virus was attempted by incubation with ethylene glycol bis(sulfosuccinimidyl succinate) (EGS; Sigma) for 1 h either on ice or at RT, at concentrations of 0.1, 0.5, 2.0, and 5.0 mM EGS. Cross-linked products were analyzed under reducing conditions by SDS-PAGE and WB as described above.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported in this study for segment 5 of the Norwegian ISAV isolate Bremnes/98 are CAD99169 (protein sequence) and AX752070 (nucleotide sequence).

RESULTS

The EB5 sequence shares high identity with previously published segment 5 sequences. Immunoscreening of the bacteriophage lambda cDNA library identified an ISAV clone designated EB5 (1,468 nucleotides). The EB5 cDNA insert contained one open reading frame of 1,332 nucleotides, theoretically encoding a protein of 444 amino acids with an estimated molecular mass of $\sim$ 48.6 kDa and a pI of 7.76. The EB5 nucleotide sequence shared 99%, 98%, and 81% identity with the previously published Scottish, Norwegian, and Canadian segment 5 sequences, respectively (9). At the amino acid level, EB5 shared 93% identity with the Scottish isolate and 91% and 78% identity with the Norwegian and Canadian isolates, respectively (9). Figure 1 shows an alignment of the EB5 and Canadian segment 5 protein sequences. No significant similarity to any other sequences was found.

Computer analyses of the EB5 sequence suggested residues M¹ to C¹⁷, F²⁷⁴ to Y²⁹², and V⁴¹⁷ to W⁴³⁹ as potential transmembrane regions (Fig. 1). Residues V⁴¹⁷ to W⁴³⁹ were predicted to be the primary transmembrane region, whereas residues M¹ to C¹⁷ were predicted to be a signal sequence. The EB5 sequence contained two potential N-glycosylation sites, two potential O-glycosylation sites, a total of 46 potential trypsin cleavage sites, and 20 cysteine residues putatively involved in disulfide bonds (all indicated in Fig. 1).

FIG. 2. Alignment of amino acid residues from the influenza A virus HA2 sequence (accession no. AF194991) predicted to have structural and sequence homology to residues G²⁸³ to Q³⁴¹ of the ISAV Bremnes/98 genomic segment 5 sequence, representing the putative coiled coil predicted by Learncoil-VMF. Conserved residues are shown with black shading.

The segment 5 protein contains a putative coiled-coil motif. Learncoil-VMF predicted a coiled-coil domain typical of viral membrane fusion proteins at residues G³⁰¹ to N³⁴⁰ of the segment 5 protein sequence with an average probability of 87% (Fig. 1). This coiled-coil domain was also predicted by COILS. Furthermore, FUGUE predicted a structural homology between residues V²⁹⁷ to K³⁶⁷ in the segment 5 protein sequence (spanning through the putative coiled coil) and the PDB structure 1jek, corresponding to the visna virus Env transmembrane core structure, with the highest z score (4.15) at 95% confidence. This permitted the construction of a 3D model of this coiled coil (not shown). Other PDB structures giving high scores were leucine zipper domains, which also form coiled-coil structures enabling oligomerization, and coiled-coil domains of the human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) gp41 proteins (data not shown).

Using PDB-BLAST, a significant structural and sequence homology was also found between residues A³⁷⁹ to M⁴²⁷ of influenza A virus HA2 (GenBank accession no. AF194991), corresponding to the coiled-coil region (8), and residues G³⁰¹ to N³⁴⁰ of ISAV segment 5, predicted to form a coiled coil (Fig. 2).

The segment 5 protein contains a putative FP. Multiple sequence alignments of the ISAV segment 5 protein sequence and viral FPs from several paramyxoviruses, orthomyxoviruses, and retroviruses suggested a putative ISAV FP (pFP) at residues C²⁷⁷ to Y²⁹² (Fig. 3). Residue G²⁷⁹ in the pFP was conserved among all the included viruses, whereas F²⁸⁰ was common to ISAV, pneumoviruses, retroviruses, and orthomyxoviruses and G²⁸⁵ was conserved in ISAV, pneumoviruses,

and retroviruses (Fig. 3). The highest level of conservation was found between the Canadian ISAV pFP (9) and the FP from bovine respiratory syncytial virus (BRSV) and between the Norwegian pFP and the human respiratory syncytial virus (HRSV) subgroup A FP. These comparisons revealed a 43.8% identity (7 of 16 residues in the pFP are identical) (Table 1). It was noteworthy that identical amino acids corresponded to residues conserved in several pneumoviruses (11). The potential transmembrane region predicted at residues F²⁷⁴ to Y²⁹² overlaps the pFP.

gp50 can be cleaved by trypsin into two disulfide-linked fragments. The antisera anti-pepEB5, anti-pepcoilEB5, and anti-EB5bac all reacted specifically with a protein of 50 kDa (gp50) in a WB analysis of purified ISAV run under reducing conditions by SDS-PAGE (Fig. 4a, lanes 1, 3, and 5). The anti-pepEB5 and anti-EB5bac antisera also reacted with two weaker bands, of approximately 30 and 100 kDa. These last two antisera also reacted with infected cells, giving identical immunostaining (described below).

A trypsin treatment of purified virus resulted in a reduction in the amount of gp50 detected by all antibodies, while the intensity of the 30-kDa protein band increased when anti-pepEB5 and anti-EB5bac were used (Fig. 4a, lanes 2 and 4). Concurrently, a band of approximately 20 kDa was detected by anti-pepcoilEB5 only (Fig. 4a, lane 6). Lowering the pH in the samples did not affect the results of reducing SDS-PAGE (results not shown). When purified virus and trypsinated purified virus were run under nonreducing conditions by SDS-PAGE, all antibodies detected only the 50- and 100-kDa proteins, indicating that the 30- and 20-kDa fragments are the cleaved products of gp50 and that these are bound together by disulfide

Virus isolate	Fusion peptide	Length (aa)	Accession #	References
ISAV Norway	-----CCGTLGI ²⁷⁷ GAWFO ²⁹² Y-----	16	CAD99169	Current paper
ISAV Canada	-----CCGTLGV ²⁷⁷ GAWFO ²⁹² Y-----	16	AF404343	(9)
HRSV	-----FICGTLGV ²⁷⁷ SAASGIAVS-----	19	P13843	(85)
BRSV	-----FICGTLGI ²⁷⁷ SAASGVAVS-----	19	AAB28458	(85)
SV5	FAGVVIGLAA ²⁷⁷ GVATA ²⁹² QVTAAVSL-----	25	AF05275	(69)
Sendai	FPGAVIGTIA ²⁷⁷ GVATS ²⁹² QIT-----	20	P04856	(37)
NDV	FIGAIGSVAA ²⁷⁷ GVATA ²⁹² QITAASL-----	25	M21881	(69)
Measles	FAGVVLGAA ²⁷⁷ GVATA ²⁹² QITAGIL-----	25	U03655	(69)
Mumps	FAGIAIGIAA ²⁷⁷ GVATA ²⁹² QVTAAVSL-----	25	AAS93903	(37)
HIV-1 NL4-3	--AVGIGALF ²⁷⁷ GTGAG ²⁹² STVGAS-----	23	VCLJBR	(17)
HIV-2 Rod	---GVFVLGF ²⁷⁷ GTAT ²⁹² CSAGAS-----	22	ENV_HV2RO	(17)
SIV tyo-1	---VPFVLGF ²⁷⁷ GTAT ²⁹² CSAGAS-----	22	ENV_SIVAT	(17)
SIV K6W	---GVFVLGF ²⁷⁷ GTAT ²⁹² CSAGAS-----	22	ENV_SIVMK	(17)
InfA HA	---GLFGAIA ²⁷⁷ ENGWEG ²⁹² IDGWYGF-----	24	HEMA_INAAI	(17)
InfB HA	---GFFGAIA ²⁷⁷ HEGGWEG ²⁹² IDGWHGY-----	24	HEMA_INBBO	(17)

FIG. 3. Overview and multiple sequence alignment of ISAV pFP (C²⁷⁷ to Y²⁹²) with known FPs of type 1 fusion proteins belonging to several viral families, including paramyxoviruses (HRSV, BRSV, simian virus 5, Sendai virus, Newcastle disease virus, measles virus, and mumps virus), retroviruses (HIV and SIV), and orthomyxoviruses (influenza A and B viruses). Fusion peptide lengths, sequence accession numbers, and references where each FP has been described are indicated. The alignment was generated with ClustalX (79). Black shading, 100% conserved residues; dark gray shading, at least 50% conserved residues; light gray shading, at least 20% conserved residues.

TABLE 1. ISAV titers after combined exposure to different concentrations of trypsin and various pHs prior to infection of cells^a

pH	Virus titer (TCID ₅₀ /ml) after exposure to the indicated concn of trypsin ^b		
	0 µg/ml	20 µg/ml	40 µg/ml
7.5	6.9 × 10 ⁵	6.9 × 10 ⁵	3.1 × 10 ⁵
5.0	6.9 × 10 ⁵	3.1 × 10 ⁵	6.9 × 10 ⁵
4.0	1.3 × 10 ⁴	2.8 × 10 ⁴	2.0 × 10 ¹

^a Trypsin and a low pH had an additive effect on the inactivation of ISAV.
^b TCID₅₀, 50% tissue culture infective dose.

bridges (results shown for anti-pepEB5) (Fig. 4b, lanes 1 and 2). A slight change in the mobility of the 100-kDa band was observed for samples treated with trypsin, possibly representing a configuration change due to trypsin cleavage.

Trimers of gp50 are detected after trypsin cleavage and exposure to low pH, 60°C, or urea. Trypsinated virus treated at pH 4.5 revealed a strong band of 150 kDa suggestive of a trimer, in addition to the 50- and 100-kDa proteins, by nonreducing SDS-PAGE (Fig. 4b, lane 3). The 150-kDa band was not detected for undigested virus after incubation at pH 4.5 (Fig. 4b, lane 4). Anti-pepcoilEB5 had a slightly reduced affinity for the 150-kDa band compared to that of anti-pepEB5 (results not shown).

The 150-kDa band representing the putative trimer was revealed at pH 5.4 to 5.6 (Fig. 4c). Exposing the polyvinylidene difluoride membrane for an extended time to Hyperfilm ECL revealed two additional bands, of approximately 75 and 125 kDa, at pH values between pH 4.0 and 7.3 (Fig. 4c). These bands probably represent intermediates of the monomer, dimer, and trimer variants of this protein.

The putative trimer was also observed in trypsin-cleaved virus treated at 60°C or with urea (Fig. 4b, lanes 6 and 8), indicating that the cleaved form of this protein is in a metastable state. Virus that had not been treated with trypsin was not susceptible to these conformational changes (Fig. 4b, lanes 5 and 7).

Chemical cross-linking using various concentrations of EGS incubated on ice or at RT did not result in increased polymerization and was not considered successful. However, 2D electrophoresis using nonreducing SDS-PAGE in the first dimension and reducing SDS-PAGE in the second dimension verified that the nonreduced 50-, 100-, and 150-kDa proteins contained the reduced 50- and 30-kDa proteins (Fig. 4d). The 150-kDa protein consisted mainly of cleaved fragments, indicating that a proteolytic cleavage of gp50 is a prerequisite for trimerization.

Trypsin and low pH are required for cell-cell fusion. Both the syncytium fusion assay and the lipid mixing assay demon-

FIG. 4. WB analyses showing specificities of antibodies towards gp50 in purified ISAV and the effects of trypsin and treatment with a kinetically destabilizing stimulus. (a) Antibody reactivity towards ISAV (lanes 1, 3, and 5) and trypsinated ISAV (tryp) (lanes 2, 4, and 6) by SDS-PAGE under reducing conditions. Antibodies are indicated at the top, and molecular sizes are indicated on the right. All antibodies reacted to ISAV gp50. Two additional weak bands, of 30 and 100 kDa, were detected by anti-EB5bac and anti-pepEB5 (lanes 1 and 3). Following trypsin digestion of ISAV, a strong 30-kDa band was detected using anti-EB5bac and anti-pepEB5, while anti-pepcoilEB5 detected a 20-kDa band in addition to gp50. (b) WB run under nonreducing conditions by SDS-PAGE. The antibodies detected only the 50- and 100-kDa proteins in both ISAV (lane 1) and trypsinated (tryp) ISAV (lane 2). Trypsinated ISAV incubated at pH 4.5 revealed an additional band of 150 kDa suggestive of a trimer (lane 3). The putative trimer was also detected in trypsinated virus treated with 4.5 M urea or incubated at 60°C (lanes 6 and 8), indicating that trypsinated gp50 is in a metastable state. The trimer was not detected in nontrypsinated virus (lanes 1, 4, 5, and 7). The antibodies used for panel a gave comparable results in this experiment, and only the results for anti-pepEB5 are presented. (c) WB analysis of trypsin-treated purified ISAV incubated at various pHs, as indicated. The putative trimer of gp50 was initially revealed at pH 5.6 as a rather weak 150-kDa protein band which increased in intensity at lower pHs. (d) Trypsin-treated purified ISAV incubated at pH 4.0 was analyzed by 2D electrophoresis run by nonreducing SDS-PAGE in the first dimension and reducing SDS-PAGE in the second dimension. The WB was immunostained with the anti-pepEB5 antiserum, confirming that the 50-, 100-, and 150-kDa protein bands could be reduced to 30- and 50-kDa proteins.

FIG. 5. Functional assays of the fusion activity of ISAV in infected ASK cells (a to d), transfected CHSE-214 cells (e and f), and infected SHK-1 cells (g to i). Cell-cell fusion was observed as syncytium formation (a and b) or as lipid mixing with subsequent dye transfer (g) in trypsinated infected cells treated with a low pH. Phase-contrast

strated that trypsin treatment combined with subsequent lowering of the pH to 5.5 or below is a prerequisite for fusion between ISAV-infected cells (Fig. 5a, b, and g). No fusion was seen when either trypsin treatment or acidification was omitted (Fig. 5c, d, h, and i). Increasing the concentration of trypsin resulted in both an increase in the total number of cells involved in syncytium formation and an increase in the number of cells involved in each syncytium (as determined by the number of nuclei). The optimal conditions for fusion were the highest concentration of trypsin possible without causing detachment of a large proportion of the cells (20 μ g/ml) and pH 4.5. This resulted in syncytia containing up to 42 nuclei in some repetitions.

Cell-cell fusion and hemadsorption in transfected cells. The optimal conditions for the syncytium fusion assay (20 μ g/ml trypsin and pH 4.5) were used to test the fusion activity in SHK-1 and CHSE-214 cells after transfection with pVAX1/EB5 and/or pVAX1/HE. Fused cells resulting in several syncytia with up to 12 nuclei were observed in wells transfected with both constructs (Fig. 5e). Syncytium formation was most abundant in wells transfected with pVAX1/EB5 and pVAX1/HE at a 2:1 ratio. No syncytia were observed in wells transfected with only HE, while on one occasion one syncytium containing 12 nuclei was observed in cells expressing only F (Fig. 5f), suggesting that the segment 5 protein has the capability of inducing fusion alone. Transfected cells incubated with 20 μ g/ml trypsin followed by incubation at pH 7.4 served as negative controls, and no signs of syncytium formation were observed in these cells expressing either protein, separately or combined. No hemadsorption activity was observed in cells expressing F (Fig. 6e and f), while cells expressing HE bound salmon erythrocytes efficiently (Fig. 6g).

gp50 is a late protein. ISAV-infected and mock-infected ASK cells were fixed every fourth hour from 4 to 24 h p.i. and were analyzed at 36 and 48 h p.i. by immunofluorescence using the anti-EB5bac rabbit immune serum in combination with the anti-HE MAb or the anti-NP MAb. No staining was observed in ISAV-infected cells until 8 h p.i., when NP was detected in the nucleus, representing an early protein as previously described (3). At 20 h p.i., NP was detected in the nucleus and cytoplasm, and HE was detected in structures resembling the endoplasmic reticulum (ER) or Golgi structure, representing a late protein, also as previously described (3). At 24 h p.i., anti-EB5bac revealed distinct staining in structures resembling the ER or Golgi structures of infected cells (Fig. 6a), and at 36 and 48 h p.i., the anti-EB5bac staining was also located at the cell membranes of infected cells (Fig. 6b). Mock-infected cells did not show staining at any time.

Following transfection of CHSE-214 cells with pVAX1/EB5 using FuGENE 6, approximately 3 to 5% of the cells expressed

microscopy pictures show the formation of syncytia containing 15 and 35 nuclei (a), and immunostaining of the same cells demonstrates the expression of the F protein (b). The F protein is visualized in green, while the nuclei are shown in blue (b, d, e, and f). The nuclei are approximately 20 μ m wide. Cell-cell fusion was also observed in trypsinated and low-pH-treated transfected cells expressing the HE and F proteins (e), and more rarely, in cells expressing the F protein alone (f). No fusion was observed when trypsin (c, d, and h) or low pH (i) was omitted. (Pictures e and f are confocal images.)

FIG. 6. Expression and localization of the F protein in ISAV-infected and -transfected fish cells (a to d) and demonstration that the F protein does not have hemadsorption activity (e to g). Immunofluorescence staining of ISAV-infected ASK cells (a and b) and transfected CHSE-214 cells (c, d, and f) shows the expression and localization of the F protein. The F protein is visualized in green, the nuclei are shown in blue (a, b, c, d, and f), and NP is visualized in red (a and b). The nuclei are approximately 20 μm wide. The first detection of the F protein using anti-EB5bac occurred at 24 h p.i., and the staining was localized to structures resembling the ER or Golgi of infected cells (a). At 36 and 48 h p.i., the F protein localized to the cytoplasm and to the plasma membrane (b [the picture was taken at 48 h p.i.]). The F protein had a similar localization pattern in transfected cells to that in infected cells, localizing to structures resembling the ER or Golgi at 24 h p.t. (c) and to the cytoplasm and plasma membrane at 72 h p.t. (d). Transmission micrographs of CHSE-214 cells which were transfected with ISAV segment 5 and incubated with Atlantic salmon (*S. salar* L.) erythrocytes demonstrate the lack of hemadsorption activity of the F protein (e). The expression of the F protein was verified by immunostaining of cells transfected in parallel with the cells shown in panel e (f), and CHSE-214 cells expressing ISAV HE served as a positive control (g). (Pictures a, b, c, and d are confocal images.)

the segment 5 protein at 5 days posttransfection (p.t.). Transfected cells were immunostained with anti-EB5bac at 24 and 72 h p.t., and cells expressing the protein revealed similar immunostaining to that of infected cells (Fig. 6c and d).

Trypsin-treated ISAV is almost completely inactivated by exposure to low pH. Incubating ISAV with 0, 20, or 40 $\mu\text{g}/\text{ml}$ trypsin followed by exposure to pH 7.3 or 5.0 prior to infecting cells had little effect on the virus titer recovered (Table 1). Lowering the pH to 4.0, however, reduced the titer >1 log. Combining the pH 4.0 treatment with a treatment of 40 $\mu\text{g}/\text{ml}$ trypsin almost completely inactivated the virus, reducing the virus titer three additional log units (Table 1).

DISCUSSION

Previous studies have localized the ISAV receptor-binding and -destroying activities (24, 43, 66). In this study, we present the characterization of the ISAV segment 5-encoded 50-kDa glycoprotein (gp50) (24) as a type I membrane fusion protein (F), adding essential information to further understand the mechanisms of ISAV pathogenicity. Type I fusion proteins require proteolytic cleavage to induce fusion competence (12), and the beneficial effect of trypsin on the replication of ISAV first implied the classification of its fusion protein (26, 41). In the present study, the requirement for proteolytic activation

was linked to the fusion activity. Cell-cell fusion assays of infected cells demonstrated the requirement for trypsin and a low pH to induce fusion, and cell-cell fusion was observed in cells transfected with F, although fusion was much more efficient in cells coexpressing HE and F. No fusion activity was observed in cells expressing only HE, and no attachment activity was observed in cells expressing only F. Previous studies have suggested that ISAV HE is an unlikely target of proteolytic activation, as indicated by the lack of disulfide binding of its cleaved products (24, 43).

The demonstration that ISAV gp50 is synthesized as a precursor (F_0 , 50 kDa), which is cleavable by trypsin to the disulfide-linked fragments F_1 (30 kDa) and F_2 (20 kDa), further implicates gp50 as the ISAV fusion protein. Two antipeptide sera defined F_1 and F_2 as the N-terminal and C-terminal cleavage products of gp50, respectively, and two theoretical trypsin cleavage sites (after residue K^{276} and after residue R^{266}) match with the size estimates of F_1 and F_2 by WB. Generally, type I fusion proteins have their proteolytic cleavage sites located next to the FP, which is usually localized to the N-terminal end of the membrane-anchored C-terminal cleavage product (12). One of the most important characteristics of membrane fusion proteins is the presence of an FP, generally a stretch of hydrophobic residues capable of interacting with and destabilizing a lipid bilayer (62). Residues F^{274} to Y^{292} gave high scores in

transmembrane predictions, reflecting the hydrophobicity of this peptide, and sequence alignments of the ISAV F protein sequence with several known viral FP sequences identified an ISAV pFP at residues C²⁷⁷ to Y²⁹². The ISAV pFP share 43.8% identity with the FPs of pneumoviruses, and the conserved amino acids reflect the conservation among several pneumoviruses (11), providing a strong indication that this represents the ISAV FP. Taking these results into consideration, we suggest that the trypsin cleavage site located after residue K²⁷⁶, which is next to the pFP, is the best candidate for the proteolytic cleavage site. Predicted glycosylation sites on both fragments implicated that the observed sizes of F₁ and F₂ by WB were in accordance with their predicted sizes of 28.2 and 18.5 kDa, respectively, if the protein were cleaved after residue K²⁷⁶.

A multiple sequence alignment of the ISAV pFP with FPs from several paramyxoviruses, orthomyxoviruses, and retroviruses revealed one residue (G²⁷⁹) that was conserved among all the viruses and two (F²⁸⁰ and G²⁸⁵) that were conserved among several viruses, corresponding to residues G³, F⁴, and G⁹ in the ISAV pFP, respectively. Additionally, a glycine corresponding to residue G⁷ (representing G²⁸³ in the protein) in the pFP was conserved between respiratory syncytial virus and ISAV. Interestingly, a study of conserved residues of paramyxovirus FPs revealed that mutations of G³ and G⁷ destabilize the native fusion protein, contributing to increased membrane fusion (37). The invariant amino acids in the FPs were suggested to preserve a balance between a high fusion activity and successful viral replication, as a high fusion activity is deleterious to cell viability. Additionally, in some paramyxoviruses these mutations influence the regulatory activity of hemagglutinin-neuraminidase (HN) on fusion activity (69). Studies of the conserved glycine residues of the FP in HIV type 1 have revealed that G¹⁰, corresponding to G³ in the ISAV pFP, seemingly the most highly conserved residue in FPs in general, is critical for both cell-cell and virus-cell fusion, while the less-conserved glycine residues are less critical (17).

The cleavability of the viral F proteins is an important determinant of pathogenicity in orthomyxo- and paramyxoviruses (reviewed in references 47, 48, and 77). Generally, F proteins with multiple basic residues at the cleavage site are cleavable by ubiquitous intracellular proteases, enabling systemic spread of the virus, thereby increasing virulence. Viruses that have a single basic residue at the F protein cleavage site require extracellular proteases for proteolytic activation and cause localized infections. In the present study, F protein sequences from two distantly related virulent ISAV strains were shown to contain single basic residues at their putative cleavage sites, indicating extracellular cleavage. Also, purified virus revealed mainly the uncleaved F protein by SDS-PAGE, and the requirement for trypsin to induce syncytia in cells suggests that the F protein is not cleaved intracellularly prior to virus assembly/budding. However, ISAV replicates efficiently in cell cultures without the addition of trypsin (16, 41), and it infects most organs in Atlantic salmon (*S. salar* L.) (55, 65), causing major pathological and histopathological changes (23, 80). Thus, in contrast to the case for other orthomyxo- and paramyxoviruses, cleavage of the ISAV F protein is not restricted by the single basic residue at the cleavage site, indicating a disparate proteolytic cleavage mechanism. Addition-

ally, the lack of multiple basic residues in the protein sequence at the cleavage sites of the distantly related ISAV isolates published suggests that the number of basic residues at the cleavage site is not as important in determining the pathogenicity of ISAV as it is for other orthomyxoviruses and paramyxoviruses (reviewed in references 10, 77, and 78). Still, the cleavability of this protein might be affected by factors such as glycosylation or an interaction with the HE.

The identification of a potential coiled coil (residues G³⁰¹ to N³⁴⁰; average probability, 87%) and the indication that it is structurally homologous to the coiled coils of influenza A virus HA2 and visna virus Env provide further evidence that ISAV gp50 is a membrane fusion protein. Coiled coils have been reported for a number of viral membrane fusion proteins, and the presence of coiled-coil motifs based on heptad repeats is among the general characteristics of type I fusion proteins (recently reviewed in references 6, 22, 57, 63, and 73). Structural homology to the visna virus Env coiled coil enabled the construction of a 3D model of the putative ISAV F coiled coil and suggested common mechanisms for the viral fusion of these viruses. The putative ISAV F coiled coil represents a possible target for antiviral peptides which potentially inhibit viral fusion by hindering the formation of the fusogenic structure, as described for retroviruses (20, 50).

The predicted 3D structure of the putative coiled-coil motif supported the formation of an F trimer similar to those characterized for influenza virus HA2 and visna virus Env proteins. The prefusion polymerization status of ISAV F₀ was not, however, established in this study, although monomers and dimers were observed by WB. However, incubating trypsin-treated purified virus at a low pH revealed a band suggestive of a trimer by WB, indicating stabilization of a trimer as part of the fusion process, as observed with other type I fusion proteins (12). This was coincident with the induction of membrane fusion activity (both requiring trypsin pretreatment and initiated at pH 5.4 to 5.6), providing a strong indication that it represents the formation of the fusogenic structure. Additionally, blotting by SDS-PAGE run in two dimensions combining nonreducing and reducing conditions verified that the 100- and 150-kDa proteins suggestive of dimers and trimers can be reduced to 50- and 30-kDa proteins, corresponding to the F₀ and F₁ fragments, respectively (F₂ was not detected by the antibody used). The trimer consisted mainly of cleaved F, probably reflecting the requirement of proteolytic cleavage for the induction of this putative fusogenic structure. The putative trimer was also detected following treatment with a high temperature or with urea, suggesting that the cleaved form of the protein is in a metastable state, in accordance with the characteristics of type I viral membrane F proteins (12). This also supports the hypothesis that the stabilization of the F trimer is associated with the formation of the fusogenic structure. The acidification of proteolytically activated virions prior to incubation on cell monolayers leads to a drastic reduction in virus titer, probably reflecting the nonreversible nature of such a conformational change, which is known to inactivate type I fusion proteins (12). Some inactivation was also observed from the acidification process alone, possibly representing a fraction of the virus population already cleaved by available proteases.

The present study is the first description of an orthomyxovirus with its fusion activity localized on a separate protein.

Influenza A and B viruses contain two glycoproteins, namely, HA, which is responsible for virus-cell attachment and membrane fusion activity, and neuraminidase (NA), which is responsible for the receptor-destroying activity. In influenza C, Thogoto, and Dhori viruses, these activities are contained in a single surface glycoprotein (48). Paramyxoviruses have an HN and a separate F protein which is different from the orthomyxovirus fusion proteins because of its pH independence and because most members of this family require a second protein to induce fusion efficiently (47). Thus, to our knowledge, the ISAV F protein represents the first described pH-dependent separate viral membrane F protein. Fusion in transfected cells indicated that ISAV HE significantly improves the membrane fusion efficacy and might be required in the fusion process. Several studies confirmed the importance of HN in influencing the fusion activity in paramyxoviruses (4, 35, 38, 47, 61), and the FP residues G³ and G⁷, which have been found to influence the regulatory effect that the paramyxovirus HN has on the fusion activity, are conserved in ISAV (69).

In conclusion, ISAV segment 5 encodes a protein that shares several biochemical properties with viral type I membrane F proteins. It is a glycoprotein (24) synthesized as a precursor, termed F₀, that is proteolytically processed into two disulfide-linked subunits, F₁ and F₂. The F₂ subunit contains a relatively hydrophobic N-terminal sequence that fulfills the criteria of an FP and a putative coiled-coil motif typical of type I fusion proteins. Furthermore, the trimerization, or more likely, stabilization of the F protein trimer, was coincident with the induction of membrane fusion activity, strongly suggesting that it represents the formation of the fusogenic structure.

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