Comparative aspects of infectious salmon anemia virus, an orthomyxovirus of fish, to influenza viruses

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Abstract Infectious salmon anaemia (ISA) is a viral disease that was first recorded in 1984 in farmed Atlantic salmon. The infectious salmon anaemia virus (ISAV) is classified as the type species of the genus *Isavirus* in the Orthomyxoviridae family and is evolutionary remote to the influenza viruses. The genome consists of eight negative single-stranded RNA segments, and it utilises the same mechanisms as influenza viruses to enter and exit cells. Although a common ancestor of ISAV and other genera of Orthomyxoviruses could be dated back several millions of years, there are still many similarities between ISAV and the influenza viruses regarding morphology, replication cycles and interactions with their respective hosts.

Keywords Salmon anemia virus · Influenza virus · Orthomyxovirus

Introduction

Comparative studies of influenza virus infections in different avian and mammalian species have a long tradition. Influenza A viruses infect many avian and mammalian species, including humans [1]. However, comparative studies to other genera of the *Orthomyxoviridae*, such as the *Thogotovirus* and *Isavirus*, have been relatively sparse. Studies of the tick borne *Thogotovirus* have yielded important comparative information regarding the pathogenesis and the host innate immune response against the orthomyxoviruses, and negative strand RNA viruses in general [2]. The infectious salmon anemia virus (ISAV), a fish orthomyxovirus, is evolutionary remote to the influenza viruses. The immune response and selection pressure which are exerted upon ISAV by its hosts are very different from those of the influenza viruses. Although the most recent common ancestor of the current genera of orthomyxoviruses may be dated back several millions of years, there are still many similarities between ISAV and the influenza viruses regarding morphology, replication cycles and interactions with the hosts. Here, information obtained from studies of a fish orthomyxovirus that possibly could be of general comparative orthomyxoviral interest, is presented.

Background

The *Orthomyxoviridae* contains five genera: influenza viruses A, B and C (FluA, B and C), *Thogotovirus* and *Isavirus*. *Thogotovirus* includes *Thogoto* and *Dhori* viruses [3], while ISAV is classified as the type species of the genus *Isavirus* [4].

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ISA was first recognized as a disease entity in 1984 in farmed Atlantic salmon (*Salmo salar*) in Norway [5] and has since been diagnosed in Canada, Scotland, the Faroe Islands, USA and Chile [6–9]. Fish in the terminal stage were found to be severely anemic, and this feature gave name to the disease. Field outbreaks of the disease have only been detected in farmed Atlantic salmon. There are no reported natural outbreaks in other species or in wild fish. The mortality can be higher than 95 % during outbreaks, but low mortality also occurs [10, 11]. The disease causes large economical losses for the fish farming industry.

Genomic organization

ISAV has a morphology resembling that of orthomyxoviruses [12–14]. Like the FluA and FluB the ISAV genome consists of eight single-stranded RNA segments of negative polarity [15–17]. The genomic segments and their corresponding encoded proteins of the different orthomyxo genera are listed in Table 1. Genetic re-assortment of ISAV has been indicated by molecular and phylogenetic sequence analysis [18]. The evolutionary distance to influenza viruses of birds and mammals is vast, as can be displayed by phylogenic relationships based upon nucleotide or amino acid sequences [4]. The amino acid identity between the ISAV proteins and those of other orthomyxoviruses is as low as 13–25% [15, 16, 19–22].

Replication

Being a virus that infects poikilothermic hosts, i.e. cold water fish, ISAV has an optimum temperature for replication of 10–15°C, and no replication is observed at 25°C or higher [34].

Each ISAV genome segment contains conserved partially self-complementary 3'- and 5'-termini that are essential for the replication process. In FluA, these structures function as promoters that interact with the polymerase complex [23]. However, compared to the 12–13 terminal nucleotides conserved in the FluA, only 8–9 nucleotides are conserved in ISAV, most likely reflecting the lower replication temperature optimum for this virus, i.e. the secondary structure of the genomic segments of ISAV requires fewer self-complementary nucleotides for stabilization [24]. As for the influenza viruses, the terminal 21–24 nucleotides are predicted to form self-complementary structures important for transcriptional regulation of viral RNA.

Some of the functions of the polymerase complex, such as cap-stealing and polyadenylation, have also been found to be present for the ISAV. The first step in ISAV mRNA synthesis is the acquisition of host cell mRNAs containing a cap structure to generate capped primers that are 8–18 nucleotides long which are then used to prime transcription of virus-specific mRNAs [24]. The cap-stealing takes place in the nucleus. Cap-stealing favors viral mRNA

Table 1 Orthomyxovirus encoded proteins and functional properties

Proteins and functions	Orthomyxoviruses				
	ISAV	FluA	FluB	FluC	THOV ¹
Polymerase subunits	PB2(1) PB1(2) PA(4)			PB2(1) PB1(2) PA(3)	
Nucleoprotein	NP(3)			NP(5)	
Receptor binding	HE(6)	HA(4)	HA(4)	HEF(4)	nd
Receptor release	HE(6)	NA(6)	NB(6)	HEF(4)	nd
Fusion	² F(5)	HA(4)	HA(4)	HEF(4)	³ gp75(4)
Matrix	M(8)	M1(7)	M1(7)	M1(6)	M(6)
Ion channel	nd	M2(7)	BM2(7)	CM2(6)	nd
Type I IFN antagonists	ORF1(7) ORF2(8)	NS1(8)	NS1(8)	NS1(7)/nd	ML(6)
Nuclear export	nd	NS2(8) (NEP)	NS2(8) (NEP)	NS2(7) (NEP)	nd
Pro-apoptosis	nd	PB1-F2(2)	–	nd	nd
Unknown structural protein	–	–	NB(6)	–	–

Abbreviations of protein names are followed by gene segment number in parenthesis. nd = not determined. ¹THOV = Thogoto virus. ²Some F proteins contain insertions near their putative cleavage site, probably acquired through a sequence based non-homologues recombination event [18]. ³gp75 of THOV has more than one function, it is a hemagglutinating and fusion protein. Modified from [50].

transcripts, and affects the cellular transcription and protein expression and thus influences the outcome of the infection. The addition of heterogeneous 5'-ends in ISAV mRNA is consistent with influenza virus mRNA synthesis, but not with that of Thogotovirus that uses only the cap structure and one or two additional nucleotides from cellular mRNAs as primer [25]. ISAV, as with the influenza viruses, has an U residue at the 3'-end of the vRNA but this U residue is lost in the cap-stealing process, and the penultimate C residue at the 3'-end of the vRNA then becomes the first nucleotide transcribed by the ISAV polymerase, which is similar to how influenza virus vRNA is transcribed [26, 27]. The cap-stealing endonuclease activity of influenza virus lies in the PA subunit of the polymerase [28, 29], and this subunit could thus be an attractive target for anti-influenza drugs. The cap-stealing endonuclease activity of ISAV has not been mapped in detail to any particular polymerase subunit.

The ISAV mRNAs are polyadenylated, by the viral polymerase complex. Polyadenylation is initiated by 13–14 signal nucleotides downstream of the 5'-end terminus of the vRNA [24]. Orthomyxoviruses are the only negative-stranded RNA viruses known to splice transcripts, which is made possible by nuclear location of the RNA replication, and accordingly, one (or may be two) groups of mRNA transcribed from ISAV segment 7 are spliced. In FluA mRNAs derived from segments 7 and 8, in FluB from segment 8 and in FluC from segment 6 and 7, can undergo alternative splicing [3].

Virulence of ISAV

ISA disease has a complex pathogenesis and virulence of ISAV is a result of interactions between virus, host and environment. Virulence of influenza viruses is dependent upon the gene constellations. Some of the genes are considered to be more central than others, i.e. the surface glycoproteins, which are responsible for receptor binding and release, and fusion of viral and endosomal membranes, are essential for pathogenesis, virulence and host tropism. For FluA and FluB receptor binding and fusion is mediated by the homotrimer hemagglutinin (HA), while release of virus progeny is mediated by the homotetramer neuraminidase (NA). FluC possesses all these functions in one molecule, the hemagglutinin-esterase-fusion protein (HEF) [3]. The surface glycoprotein HA is the major determinant of virulence in FluA [30]. HA is produced as a precursor molecule HA0, which has to be post-translationally cleaved into two subunits, HA1 and HA2, for the virus to become infectious. HA1 binds to 5-N-acetylneuraminic acid on cell receptors, while HA2 mediates fusion of the viral and the endosomal membranes. The cleaving of HA0

leads to exposure of the fusion peptide at the hydrophobic N-terminus of HA2 which is inserted into target membranes after acidification of the endosome.

In low pathogenic avian influenza viruses (LPAIVs) the HA0 cleavage is performed by extracellular trypsin-like proteases in the epithelium of the intestinal and respiratory tract. The acquisition of multiple basic amino acids at the HA0 cleavage site in highly pathogenic avian influenza viruses (HPAIVs) of the H5 and H7 subtypes, makes it possible for ubiquitous furin-like proteases to cleave HA0 and thus facilitates replication of HPAIV in multiple tissues [31].

A deletion of 19 amino acids have been found to occur in the stalk portion of the NA of H5N1 chicken viruses that differ from H5 viruses of wild aquatic birds. The stalk deletion was found to decrease the ability of NA to release virus progeny from cells. Comparison of amino acid sequences of the HA and NA of viruses obtained from different avian species revealed that additional glycosylation of the HA and a shortened NA stalk are characteristic features of the H5 and H7 chicken viruses. These mutations are suggested to be markers for virus' adaptation in poultry, but it is not known if the deletion is related to pathogenicity [32, 33].

ISAV has been demonstrated to possess the major functional characteristics of the orthomyxoviruses associated with virulence and pathogenesis: hemagglutinating (i.e. receptor binding), receptor release and fusion activities. However, these activities are associated with two surface proteins called the hemagglutinin-esterase (HE) and the fusion (F) glycoproteins [14].

ISAV hemagglutinates erythrocytes from several fish species including Atlantic salmon, but the receptor release activity that dissolves the hemagglutination is not doing this for Atlantic salmon erythrocytes [34]. The receptor release activity is analogue to the neuraminidase activity of influenza virus. The inability of HE to dissolve the hemagglutination of salmon erythrocytes may be of importance for ISAV virulence and pathogenesis. The ISAV HE is not cleaved in two subunits as the influenza HA, however the F protein must be cleaved in order for the virus to be infectious [35]. A comparison of the major surface glycoproteins of ISAV and FluA are displayed in Fig. 1.

The ISAV F protein, which was the first described separate pH-dependent viral membrane F protein, shows several characteristics of a type I fusion protein. It is synthesized as a precursor F0 that is activated by proteolytic cleavage into two disulfide-linked subunits, F1 and F2 [35]. The coiled-coil domains, trimerization, the hydrophobic N-terminal sequence of F2, and the requirement for a low pH to induce fusion, are other criteria of type I fusion proteins [3, 13, 36]. Analysis of the gene encoding the ISAV fusion protein has revealed insertions in several virus

Fig. 1 Major surface glycoproteins of FluA and ISAV

The illustration shows monomers of HA, NA, HE and F proteins. In FluA, the HA which is responsible for receptor-binding and fusion, is cleaved into two disulfide-linked subunits to gain viral infectivity. Receptor-release is associated with NA. In ISAV the fusion protein F being a separate surface glycoprotein, is cleaved into two disulfide-linked subunits. Receptor-binding and release activities are found in HE.

isolates, in proximity to the protein's putative cleavage site [18], suggesting the presence of a recombinational hot spot. It could be speculated that this could lead to alterations in the cleavage specificity of the fusion protein, however, no specific motifs similar to those seen in the FluA HA, which exerts the fusion activity, of HPAIV have been found.

In FluA HA most of the variability is located in distal parts of the molecule driven by the selective pressure of the humoral response [3]. The variation in the distal part of the HE molecule in ISAV is much more modest [37], indicating a lower selective pressure on the ISAV HE by the salmon humoral response. Although Atlantic salmon produce neutralizing antibodies against the ISAV, the neutralization titres are generally low [38]. The salmon humoral response does not include an affinity maturation similar to that of mammals, and the response is dominated by IgM-like complement fixing antibodies. The low neutralization activity of the salmon sera implies that the interference of the salmon antibodies with the virus' binding to the receptors are not as distinctive as for the humoral response in mammals. This may also be reflected in differences in glycosylation pattern of ISAV and FluA, where there is lower glycosylation of ISAV HE ectodomain, with two N-linked oligosaccharide chains per subunit, compared to between three and nine (depending on the isolate) of the ectodomain of FluA HA.

However, most of the variability within the ISAV HE is concentrated to a small highly polymorphic region (HPR) near the transmembrane domain. This region is characterized by the

presence of gaps, rather than single-nucleotide substitutions [39]. The polymorphism in this region has been suggested to result from deletions, or possibly recombinations, of a full-length precursor gene (HPR0) as a consequence of strong functional selection. The HPR0 type is non- or low virulent, and has never been found in outbreaks of ISA disease, but as subclinical infection in healthy salmon. The transition from wild fish living more or less solitarily to the high density of single species in farming could be a prerequisite for the functional selection. The HE HPR is thus considered to represent an important virulence marker.

The reservoir for FluA is wild birds [1]. The reservoir for ISAV is assumed to be wild salmonid fish [17]. In both the aquaculture and poultry industries a large number of individuals of a single species are confined to limited space, which is a favorable condition for virus transmission. Some viral mutants have better fitness than others and the viral fitness is generally increased by large population transmissions [40]. The similarities between pathogenic ISAV and HPAIV regarding inserts of nucleotides adjacent to the proteolytic cleavage site of the fusion peptide and truncation of the stalk of the receptor release protein are intriguing.

Interplay with innate immune response

Both ISAV and influenza viruses encode proteins with antagonistic effect against interferon type I. The gene

products of ISAV segments 7 and 8 (s7ORF1 and s8ORF2) interfere with the innate immune response. Although both products differ in characteristics, the s7ORF1 is non-structural, primarily localized in the cytoplasm and unable to bind RNA while the s8ORF2 is structural, predominant localized in the nucleus and able to bind RNA, both can antagonize the interferon type I response [41, 42]. In FluA segment 8 encodes the non-structural IFN-antagonist NS1 [43]. Sensitivity to the inhibitory actions of Mx GTPases is a characteristic property of all orthomyxoviruses [2]. There is considerable conservation among Mx proteins from different vertebrate species.

Pathogenesis

ISA is a systemic disease attended with viremia and it affects mostly the circulatory system. The major target cells for virulent ISAV are endothelial cells lining blood vessels of all organs, including sinusoids, endocardium and endothelial macrophages [44–46]. The gills are assumed to be the major port of virus entry, but oral entry cannot be excluded [47, 48]. Non-virulent ISAV with a HE of the HPR0 variant is often found to present in the gills and to a lower extent in other organs.

The port of virus entry of LPAIV in chickens is the nasal cavity with subsequent distribution to the respiratory and intestinal tracts. The replication of HPAIV H5N1 in chickens starts in the nasal epithelium, and induces submucosal inflammation. The virus also replicates in inflammatory cells, principally macrophages and heterophils as well as in capillary endothelial cells. This causes dissemination through the vascular or lymphatic systems and replication in a variety of parenchymal cells types within different organs [49].

Conclusion

The H1N1 (2009) pandemic, known as “swine flu”, the HPAI H5N1 epidemic and the emergence of ISAV in Atlantic salmon are of highly topical interest. In this short review common features of ISAV and influenza viruses, including virion structure, strategy of replication, agglutination of erythrocytes, and presence of receptor release activity have been highlighted.

Replication mechanisms that have been preserved throughout the evolution could be considered vital for the virus, and perhaps in the future be utilized as possible universal targets for immunological or therapeutical interactions. Due to the vast evolutionary distance, adaption to different intracellular environments and immune responses

one should, however, be cautious not to draw extensive conclusions from comparative studies. However, ISAV do not infect humans and could therefore be considered as a safe alternative model in general orthomyxovirus research in the future.

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