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DOI: https://doi.org/10.1128/JVI.02516-14

JVI Accepts, published online ahead of print on 15 October 2014
J. Virol. doi:10.1128/JVI.02516-14

Swine IFITM proteins potently inhibit influenza A virus replication

Running title: Antiviral activity of swine IFITM proteins

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word counts:

abstract: 75 words

main text: 1776 words

Abstract

Human IFITMs were identified as restriction factors of influenza A virus (IAV). Given the important role of pigs in the zoonotic cycle of IAV we cloned swine IFITMs (swIFITM) and found two IFITM1-like proteins, one homologue of IFITM2 and one of IFITM3. We show that swIFITM2 and swIFITM3 localize to endosomes and display potent antiviral activity. Knockdown of swIFITMs strongly reduced virus inhibition by interferon establishing the swIFITMs as potent restriction factors in porcine cells.

Main text

The **i**nter**f**eron-**i**nducible **t**rans**m**embrane proteins (IFITMs) were identified as potent inhibitors of different viruses, including influenza A virus (IAV), West Nile Virus, SARS coronavirus and others (1-5). In particular, human and mouse IFITM3 have been studied regarding their antiviral potential but also the mechanism of viral restriction. *Ifitm3* knockout mice showed accelerated disease progression and higher morbidity and mortality upon IAV infection (6). Moreover, it was found that humans with a single nucleotide polymorphism in the *ifitm3* gene have a significantly higher risk for a severe course of IAV infection (6, 7). The antiviral mechanism of the IFITMs is not yet fully understood but it has become clear that viral fusion is targeted by these proteins (8-11). While we have a good understanding of the antiviral potential of human and mouse IFITMs, much less is known about the activity of IFITMs in other species. Given the important role of pigs in the zoonotic cycle of IAV we aimed to elucidate the antiviral potential of swine IFITM (swIFITM) proteins.

We cloned swIFITMs from cDNA obtained from interferon (IFN)-stimulated pig cells according to sequences deposited in the NCBI database. We were able to amplify the porcine homologues of human IFITM1, IFITM2 and IFITM3. For IFITM1, two porcine homologues that differ in their N- and C-termini, named swIFITM1a and 1b, were found. Swine IFITM5 could not be amplified out of cDNA and was thus synthesized. The amino acid alignment of the IFITM proteins from humans, pigs and chickens shows that the IFITMs display certain features that are conserved across all three species, such as the two transmembrane domains, the palmitoylation sites at positions 73 and 74 or the ubiquitination sites at positions 85, 90 and 106 (Fig.1A) (12-14). Moreover, swine and human IFITM2 and IFITM3 share an N-terminal extension of about 20 amino acids that contains the endocytosis motif YEML, which the IFITM1 proteins of both species lack (Fig.1A) (15, 16). This alignment suggests that the swine IFITM proteins could have similar antiviral properties as their human counterparts.

The induction of human IFITM1-3 upon IFN stimulation or virus infection is well-established (1, 17). While Miller *et al*. showed that in pigs swIFITMs are induced in tracheobronchial lymph nodes upon infection (18), a direct induction of swIFITMs upon IFN treatment has not been established to date. We thus tested the inducibility of swIFITMs upon IFN stimulation or IAV infection in porcine cell lines. We stimulated porcine NpTr, PK-15 or NSK cells with universal IFN (1000 U/ml) for 24h or infected with A/WSN/33 (WSN) (MOI 0.01) for 24h or 48h (19). RT-qPCR with specific primers for the individual swIFITMs (primer sequences available upon request) revealed a pronounced upregulation of swIFITM1a, swIFITM1b, swIFITM2 and swIFITM3 upon IFN-stimulation or infection in NSK as well as PK-15 cells (Fig.1B, C). In contrast, swIFITM transcripts were induced less than 3-fold in NpTr cells (Fig.1D). Moreover, when we treated NpTr cells with strong inducers of the IFN response, such as IAV lacking NS1, poly(I:C) or a preparation of Sendaivirus that contains large amounts of defective interfering particles, we detected only low levels of swIFITM induction (Fig.1E). This was not limited to swIFITMs; also swine MX1 and swine OAS, both known to be upregulated by IFN, displayed only low levels of induction. This suggests that the NpTr cells are not able to mount a strong IFN response. Despite these differences in the induction of the swIFITMs, all three cell lines were permissive for robust replication of IAV (Fig.1F). Expression of swIFITM5 could not be detected in any of the cell lines (data not shown). These results suggest that like their human counterparts, swIFITM1-3 constitute a first line of defence against viral infections.

Next, we analyzed the subcellular localization of the swIFITMs. We transiently transfected human A549, swine NpTr or swine NSK cells with constructs encoding flag-tagged human or swine IFITMs. IFITM proteins were stained using an antibody against the flag-tag, while late endosomes were marked using an antibody recognizing lysobisphosphatidic acid (LBPA) (20). Confocal microscopy revealed that the subcellular localization of porcine IFITMs is similar to their human homologues, with swIFITM1a and 1b localizing predominantly to the plasma membrane while swIFITM2 and 3 accumulate in late endosomes. This is illustrated by their co-localization with the

late endosomal marker LBPA in representative images (Fig.2A) and the quantification of co-localization from 8-12 cells per construct and cell line (Fig.2B). Of note, human and swine IFITM2 displayed the highest degree of co-localization with LBPA. The observed localization for the human IFITMs is in agreement with previous reports for overexpressed but also for endogenous human IFITMs validating our approach (8). Moreover, the endosomal localization of human and swine IFITM2 and 3 confirms the importance of the endocytosis motif in the N-terminus for pronounced localization to endosomes (15, 16). When we analyzed the localization of the swIFITM proteins during IAV infection we did not detect changes in their intracellular distribution compared to uninfected cells (Fig.2C).

To evaluate the antiviral potential of swIFITMs we performed an IAV reporter assay in cells transiently transfected with the different swIFITMs. The reporter plasmid encodes luciferase in complementary reverse orientation flanked by IAV non-coding regions, preceded by a human or swine pPolI promoter for use in human or porcine cells, respectively. This assay provides the advantage that only reporter activity from transfected cells is measured, enabling us to assess the impact of the different IFITM proteins on IAV in a transient transfection system. The construct for human cells has been used before (21) and, for porcine cells, we adapted a construct described previously by introducing three mutations at positions 3, 5 and 8 in the promoter region (22, 23). After transfection with the reporter plasmid plus the indicated IFITM-encoding plasmid, we infected with IAV A/WSN/33 at an MOI of 0.1 in HEK293-T and an MOI of 1 in NpTr cells and measured luciferase production 24h post infection (p.i.). We observed that swIFITMs show a dose-dependent restriction of IAV in HEK293-T cells, with swIFITM2 and 3 being the most antivirally active and their activity being comparable to huIFITM3 (Fig.3A). However, it should also be noted that swIFITM2, swIFITM3 and huIFITM3 were expressed to higher levels than the other IFITMs, which is in line with the dose-dependency of restriction. We then confirmed the antiviral activity of swIFITMs in porcine cells, with swIFITM2 showing the most potent restriction

of IAV (Fig.3B). These data from the transient transfection assay suggest that the swIFITM proteins possess antiviral activity.

In order to corroborate these findings, we generated stable cell lines overexpressing the different swIFITMs in the background of the porcine NpTr and NSK cells by lentiviral transduction. Cells were subcloned until more than 99% of cells were positive for expression. To test the antiviral activity of overexpressed swIFITMs we infected the cell lines with either the human strain A/HongKong/68 (MOI 1), or the swine isolate A/swine/Zurich/25/06 (MOI 1). At 48h p.i., supernatants were harvested and viral titers determined by plaque assay. Expression of the swIFITMs reduced viral titers of IAV in the NpTr cell lines, with swIFITM2 being the most potent and reducing titers around 1000-fold (Fig.3C, D). It should be noted that we were not able to generate stable NpTr cell lines expressing high levels of swIFITM3 and 5 (Fig.3E), which could contribute to the reduced potency of swIFITM3 compared to swIFITM2. In the NSK cell lines, we also observed potent inhibition of IAV replication upon expression of the different swIFITMs, except for swIFITM1b (Fig.3F, G). Again, swIFITM2 displayed the most potent antiviral activity. As in the NpTr cells, we were not able to generate cell lines expressing high levels of swIFITM3 or swIFITM5. These data suggest that high expression levels of these two swIFITM proteins are not well tolerated in porcine cells. The differences in expression levels make a direct comparison of the antiviral activity difficult since we observed in the transient transfection assay in 293T that higher expression levels lead to better inhibition of IAV. However, when comparing swIFITM2 to swIFTIM1a or 1b, expression levels are similar but swIFITM2 is the most active. Moreover, swIFITM3 despite being expressed at much lower levels than swIFITM1a or 1b has comparable activity. We therefore suggest that swIFITM2 and swIFITM3 which localize predominantly to late endosomes display the strongest antiviral potential.

Since the assays described above were performed with overexpressed swIFITMs, we next assessed whether endogenous swIFITMs also reduce viral replication. To this aim, porcine NSK

cells were transfected with two different siRNAs targeting swIFITMs (siIFITM #1, siIFITM #2), a positive control siRNA against the viral nucleoprotein NP or a negative control non-targeting siRNA. At 36h post transfection, cells were either mock-treated or treated with universal type I IFN (1000 U/ml). At 12h post treatment, cells were infected with IAV A/WSN/33 (MOI 0.0001), supernatants were harvested at 24h p.i. and virus titers were determined by plaque assay. In the absence of IFN, knockdown of swIFITM had no effect on virus titers (Fig.4A grey bars). However, in IFN-treated cells, viral titers were increased by 10-fold when swIFITMs were downregulated (Fig.4A black bars). To confirm that knockdown by siRNA was successful we measured mRNA levels for the different swIFITMs and swMX1 in parallel samples and found that both siRNAs designed to target all IFN-induced swIFITMs potently downregulated their expression, whereas swMX1 as a control IFN-stimulated gene was not affected (Fig.4B, C). Knockdown of swIFITMs by the two different siRNAs was further confirmed at the level of protein. Since detection of endogenous porcine swIFITM proteins was unsuccessful using available antibodies for human IFITMs we tested the siRNAs in the NSK cell lines overexpressing swIFITMs and confirmed knockdown (Fig.4D). Downregulation of viral NP levels by the NP-specific siRNA was also confirmed by western blot (Fig.4E). These data show that swIFITMs constitute an important part of the IFN response against IAV in porcine cells and confirm our results obtained with overexpression of swIFITMs.

In summary, we show that swIFITM1-3 are upregulated by type I IFN and IAV infection in porcine PK-15 and NSK cells. While swIFITM1a and 1b displayed prominent plasma-membrane localization, swIFITM 2 and 3 were found to co-localize with a marker for late endosomes. Over-expression of swIFITMs resulted in reduced influenza virus reporter activity and reduced viral titers. Although different expression levels of the swIFITMs make a direct comparison of the antiviral potencies difficult the data suggest that swIFITM2 and swIFITM3 display the strongest antiviral activity. Importantly, we also show that endogenous swIFITMs constitute a large part of the antiviral activity of IFN in porcine cells. In future studies, it will be interesting to characterize

the IFITMs from different swine breeds for their antiviral potential and thereby help to select pig breeds with increased resistance to IAV infections.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (31003A_135278) and the Novartis Research Foundation to SSt. We would like to thank Prof. Georg Kochs (University Hospital Freiburg, Germany) and Dr. Monika Engels (University of Zurich, Switzerland) for providing porcine cell lines and swine influenza viruses and Prof. Wendy Barclay (Imperial College London, UK) for the influenza virus reporter construct for porcine cells. Imaging was performed with support of the Center for Microscopy and Image Analysis, University of Zurich.

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Figure legends

Figure 1. Amino acid alignment of human, swine and chicken IFITM proteins and induction of swine IFITMs upon IFN stimulation or virus infection. (A) Amino acid alignment of human, swine and chicken IFITM1, IFITM2, IFITM3 and IFITM5. Functional motifs and domains are highlighted with colored boxes. Abbreviations: hu human, sw swine, ch chicken, TM transmembrane domain **(B-D)** Induction of swIFITM expression by IFN or IAV infection. NSK **(B)**, PK15 **(C)** or NpTr **(D)** porcine cells were infected with A/WSN/33 at an MOI of 0.01 or treated with universal IFN (1000 U/ml) for 24h. At the indicated times post treatment, total mRNA was extracted and used to perform RT-qPCR using specific oligos for the different swIFITMs or swMX1 as positive control. Fold-changes were calculated according to the Pfaffl method using swGAPDH mRNA as reference. A representative experiment from two biological replicates, each performed in triplicate, is shown with error bars representing the 95% confidence interval. Statistical significance between IFN-treated or IAV-infected cells and control untreated cells was assessed by an unpaired two-tailed Student's t test (*, $P<0.05$). **(E)** NpTr cells were transfected with poly(I:C) or infected with A/PR/8/34ΔNS1 (ΔNS1) or Sendaivirus (SeV) for 24h. Total mRNA was extracted and used to perform RT-qPCR using specific oligos. Fold-changes were calculated according to the Pfaffl method using swGAPDH mRNA as reference. A representative experiment is shown with error bars representing the 95% confidence interval. Statistical significance between poly(I:C)-treated or infected cells and control untreated cells was assessed by an unpaired two-tailed Student's t test (*, $P<0.05$). **(F)** IAV replication in porcine cell lines. NSK, PK-15 and NpTr cells were infected with A/WSN/33 as described in B-D and viral titers in the supernatant at 24/48h p.i. were determined by plaque assay. The dashed line indicates the limit of detection.

Figure 2. Localization of human and swine IFITM proteins in human and porcine cells. (A) Representative confocal images of human A549 **(a-g)**, porcine NpTr cells **(h-k)** or porcine NSK

cells **(l-o)** transfected with flag-tagged human or porcine IFITM-encoding constructs are shown. IFITMs were stained using an antibody against the flag-epitope (red), late endosomes using an antibody against the late endosomal marker LBPA (green) and cell nuclei were marked using DAPI (blue). Cells were imaged using a 63x objective and a magnification of 3x (scale bar = 10 μm) **(B)** Co-localization of IFITM proteins with the late endosome marker LBPA was quantified. Horizontal bars represent the mean of the Pearson's correlation coefficient (Rr) calculated for 8-12 cells using ImageJ, with error bars marking the 95% confidence interval. Statistical significance of increased co-localization for the indicated IFITM construct compared to huIFITM1 or swIFITM1a, respectively, was assessed by an unpaired two-tailed Student's t test (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). **(C)** Representative confocal images of NSK cells transfected with flag-tagged porcine IFITM-encoding constructs and infected with A/WSN/33 at an MOI of 5 are shown. IFITMs were stained using an antibody against the flag-epitope (red), infection was controlled using an antibody against IAV NP (green) and cell nuclei were marked using DAPI (blue). Cells were imaged using a 63x objective and a magnification of 3x (scale bar = 10 μm).

Figure 3. Expression of swIFITMs potently inhibits IAV. (A-B) Transient transfection assays. HEK293-T **(A)** and NpTr **(B)** cells were transfected with an IAV-dependent firefly luciferase reporter construct and increasing amounts of plasmids encoding flag-tagged swine IFITMs. At 24h post transfection, cells were infected with A/WSN/33 at an MOI of 0.1 **(A)** or 1 **(B)**. At 24h p.i., cells were lyzed and firefly luciferase activity measured. Data were normalized to luciferase activity in cells transfected with an inactive Mx1 mutant (neg con), while murine Mx1 and human IFITM3 served as positive controls (pos con). Mean values from three biological replicates, each performed in triplicate, are shown with error bars representing standard deviation. Statistical significance between cells transfected with swIFITMs and cells expressing the negative control was assessed by an unpaired two-tailed Student's t test (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). Expression levels of swine IFITM proteins were confirmed

296 by western blot, quantified using Multi Gauge software and expression levels in relation to human
297 IFITM3 expression are given below the western blots. **(C-H) Stable cell lines expressing**
298 **swIFITMs.** NpTr **(C, D)** or NSK **(F, G)** cells stably overexpressing flag-tagged swIFITM proteins
299 were infected with A/HongKong/68 (MOI 1) **(C, F)** or A/swine/Zurich/25/06 (MOI 1) **(D, G)**. At 48h
300 p.i., supernatants were harvested and virus titers determined via plaque assay on MDCK cells.
301 Mean values from three replicates are shown with error bars representing standard deviation.
302 Statistical significance between titers from cells expressing swIFITM proteins and cells
303 transduced with the empty vector (con) was assessed by an unpaired two-tailed Student's t test
304 (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$). The dashed lines indicate the limit of
305 detection. Expression levels of swIFITMs were confirmed by western blot, quantified by Multi
306 Gauge software and expression levels relative to the strongest band are given below the western
307 blots **(E, H)**.

308 **Figure 4. Endogenous swIFITMs contribute to the antiviral activity of IFN in porcine cells.**
309 **(A)** NSK cells were transfected with two siRNAs targeting swIFITMs (#1 and #2) or the viral
310 nucleoprotein (NP) or a non-targeting control siRNA. At 36h post transfection, cells were either
311 left untreated or treated with universal type I IFN (1000 U/ml). At 12h post treatment, cells were
312 infected with A/WSN/33 (MOI 0.0001) and supernatants were harvested at 24h p.i. Viral titers
313 were determined by plaque assay. Mean values with standard deviation from three replicates are
314 shown. The dashed line indicates the limit of detection. **(B, C)** A parallel set of samples was
315 siRNA-transfected and mock or interferon-treated as above. At 48h post transfection, cells were
316 harvested and mRNA levels of the different swIFITMs and swMX1 were determined by RT-qPCR.
317 Fold-changes were calculated according to the Pfaffl method using swGAPDH mRNA as
318 reference. Mean values with the 95% confidence interval from three replicates are shown.
319 Statistical significance between NP- or swIFITM-silenced cells and cells transfected with the non-
320 targeting siRNA was assessed by an unpaired two-tailed Student's t test (*, $P<0.05$). **(D)** Stable
321 NSK cells expressing swIFITMs were transfected with either an siRNA targeting swIFITMs or a

non-targeting control siRNA (con). At 48h post transfection cells were lyzed and the amount of flag-tagged swIFITMs assessed by western blot. Results for two different swIFITM-specific siRNAs (#1 and #2) are shown. **(E)** NSK cells were transfected with either an siRNA targeting IAV NP or a non-targeting control siRNA (con) . At 36h post transfection cells were treated with universal type I IFN (1000 U/ml) and 12h post treatment cells were infected with A/WSN/33 (MOI 0.0001) as in 4A. At 24h p.i. cells were lyzed and NP levels assessed by western blot.