Genetic Screen of a Library of Chimeric Poxviruses Identifies an Ankyrin Repeat Protein Involved in Resistance to the Avian Type I Interferon Response

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ABSTRACT

Viruses must be able to resist host innate responses, especially the type I interferon (IFN) response. They do so by preventing induction or activity of IFN and/or by resisting the antiviral effectors it induces. Poxviruses are no exception, with many mechanisms identified whereby mammalian poxviruses, notably vaccinia (VACV) but also cowpox and myxoma viruses, are able to evade host IFN responses. Similar mechanisms have not been described for avian poxviruses (avipoxviruses). Restricted for permissive replication to avian hosts, they have received less attention; moreover the avian host responses are less well characterised. We show that the prototypic avipoxvirus, fowlpox virus (FWPV) is highly resistant to the antiviral effects of avian IFN. A gain-of-function genetic screen identified fpv014 as contributing to increased resistance to exogenous recombinant chicken IFN-α (ChIFN1). Fpv014 is a member of the large family of poxvirus (especially avipoxvirus) genes that encode proteins containing N-terminal ankyrin repeats (ANKs) and C-terminal F-box-like motifs. By binding the Skp1/Cullin-1 complex, the F-box in such proteins appears to target ligands bound by the ANKs for ubiquitination. Mass spectrometry and immunoblotting demonstrated that tandem affinity-purified, tagged fpv014 was complexed with chicken cullin-1 and Skp-1. Prior infection with an fpv014 knockout mutant of FWPV still blocked transfected poly(I:C)-mediated induction of the IFNβ (ChIFN2) promoter as effectively as parental FWPV, but the mutant was more sensitive to exogenous ChIFN1. Therefore, unlike the related protein fpv012, fpv014 does not contribute to the FWPV block to induction of ChIFN2, but does confer resistance to an established antiviral state.
The major mammalian innate host response to virus infection is the type I interferon (IFN) system, consequently many viruses have evolved mechanisms to evade or subvert it (1, 2). The prototypic mammalian poxvirus, vaccinia virus (VACV), encodes a number of proteins that have been shown to modulate the IFN system in diverse ways (reviewed by Perdigueró et al. (3)). They include double-stranded (ds) RNA-binding protein E3 (4), eIF-2α mimic K3 (5), soluble IFN receptor mimic B18 (6, 7), Stat phosphorylation inhibitor H1 (8) and NF-κB activation suppressor K1 (9). With the exception of H1, no type I IFN modulators have yet been identified for the avipoxviruses.

The genomes of the avipoxviruses are larger than those of the mammalian poxviruses. Although the genomes of two avipoxviruses, Fowlpox virus (FWPV; type species of the Avipoxvirus genus) and Canarypox virus (CNPV), have been sequenced (10, 11), there are no obvious orthologs of the VACV IFN modulators (such as E3L, K3L and B18R), with the exception of an ortholog of H1 (12). The lack of E3L and K3L is a feature not unique to avipoxviruses - they are also absent from the now only remaining extant human poxvirus, molluscum contagiosum virus, which causes chronic infection (13). Avipoxviruses also lack members, several of which are immunomodulatory (14-20), of the family of proteins found in VACV that share a structural fold with host apoptosis-controlling Bcl-2 (17). Avipoxviruses do encode potential orthologs of immunoregulators less frequently found in mammalian poxviruses, such as the IL-10 analogue encoded by CNPV ((11) and found in mammalian poxviruses other than VACV, e.g. Orf virus (21)) and TGF-β orthologs found initially in FWPV.
(10) and CNPV (11) then subsequently in deerpox virus (22). Ligand-binding studies have also identified a binding protein for chicken IFN-γ, distinct from IFN-γ-binding proteins encoded by mammalian poxviruses (23).

We show here that FWPV is highly resistant to chicken type I IFN (ChIFN), considerably more so than the artificially chicken cell-adapted strain of VACV, modified virus Ankara (MVA) and that the resistance is due to FWPV encoded protein(s). We have used a broad-scale genetic screen to search for modulators, encoded by the FWPV genome, involved in subverting the IFN response. This 'gain-of-function' screen involved construction of a library of chimeric MVA viruses, each carrying 4 or 8 kbp segments of the FWPV genome, which were then screened to identify a gene(s) conferring enhanced resistance to chicken type I IFN. The screen identified a member of a large poxvirus gene family that is particularly extensive in avipoxviruses, members of which had not been previously associated with subverting the IFN response.

**MATERIALS AND METHODS**

**Cells and viruses**

Primary chicken embryo fibroblasts (CEF), derived from specific pathogen free (SPF)-quality 10-day old embryos, and provided by the Institute for Animal Health (Compton, Berkshire, U.K.) were grown in 199 medium supplemented with 10% tryptone phosphate broth (TPB), 10% newborn bovine serum, nystatin and penicillin/streptomycin. Immortalised chicken fibroblast DF-1 cells (24) sourced from the American Tissue Culture Collection (ATCC), were
maintained in DMEM supplemented with 10% Foetal Bovine Serum (Autogen Bioclear) and penicillin/streptomycin.

Throughout this study we have used the previously described, attenuated FWPV strain FP9, as well as its pathogenic European progenitor, HP-1 (25). VACV VTN, a Western Reserve (WR) recombinant virus expressing the porcine transmissible gastroenteritis virus (TGEV) nucleoprotein in the thymidine kinase (TK) locus (26), was a gift from P. Britton (Institute for Animal Health, Compton U.K.). Parental VACV MVA (II/85), from laboratory stocks, was originally provided by Anton Mayr (Ludwig-Maximilians University, Munich, Germany). Recombinant VACV MVA-LZ, derived by insertion of the empty β-galactosidase selection plasmid, pSC11, at the TK locus, and known originally as MVA-SC11 (27), was provided by S. Gilbert, University of Oxford, U.K. The avirulent A7(74) strain of Semliki Forest virus (SFV) was donated by J. Fazakerley (University of Edinburgh, U.K.).

Plasmids

The ChIFN2 promoter reporter (pChIFN2lucter (28)) and the constitutive β-galactosidase reporter plasmid (pJATlacZ (29)) have been previously described, as has pEFPlink2, which uses the elongation factor 1α promoter to direct high levels of expression of cloned cDNAs, as originally described by Marais et al. (30).

Transfection of cells with poly(I:C) and assay of luciferase reporters

Chicken DF-1 cells in 12-well plates were transfected with the chicken IFN-2 promoter reporter (pChIFN2lucter) and the constitutive reporter plasmid...
pJATlacZ. Sometimes they were additionally transfected with mammalian expression vector pEFPlink2 driving the overexpression of viral proteins, or empty as a control vector. Following recovery for 24 hours cells were either left uninfected or infected with either wild type fowlpox virus (FP9) or knockout virus at an MOI of 10. Following infection for 4 hours, cells, when appropriate, were transfected with poly(I:C) (10µg/ml) using Polyfect (Qiagen) or Lipofectamine (Invitrogen), as described by Childs et al. (28), and incubated for 16 hours. Luciferase assays were carried out and data were normalised using β-galactosidase measurements.

Chicken IFN-α

Preliminary studies were undertaken using recombinant chicken IFN-α (ChIFN1, (31), P. Staeheli, University of Freiburg, Germany). Subsequently the ChIFN1 gene was amplified from CEF DNA by PCR and cloned it into pEFPlink2 using Ncol and Xbal sites. This plasmid was transfected into 293 cells (9cm plate); the supernatant, collected after 48 h incubation, was centrifuged to remove cell debris and was titrated by 50% plaque reduction assay (32) using SFV A7(74) on CEF.

Construction of FWPV-chimeric MVA

The approach adopted, after considerable optimisation of the individual steps, involved using Splice-Overlap-Extension (SOE) PCR to assemble 10 kbp linear recombination templates from four constituent parts: (i) 8 (or 4 kbp) PCR fragments of the FWPV genome (amplified using high fidelity Taq polymerase), flanked to the left by (ii) MVA sequences (20406 to 20894), to the proximal right
by (iii) a VACV p7.5 promoter upstream of the *E. coli* *gpt* gene and to the distal right by (iv) MVA sequences (20916 to 21472). Parts (iii) and (iv) were pre-assembled by SOE PCR before assembly of the complete linear recombination template from the resulting three components. The templates were purified by agarose gel electrophoresis, where crystal violet was used as the stain, rather than ethidium bromide, to avoid UV damage to the long templates.

The assembled FWPV genomic DNA selection cassettes were transfected into DF-1 cells previously infected with an MVA recombinant, MVA-LZ (which carries the *E. coli* lacZ gene inserted into the MVA thymidine kinase locus under control of the VACV p11 promoter to facilitate plaque identification by staining for β-galactosidase). Recovered viruses were bulk passaged three times in CEF under mycophenolic acid selection (in the presence of xanthine and hypoxanthine) for the *gpt* gene. Viral genomic DNA was then extracted and analysed by PCR to confirm recombination of the FWPV genomic insert and loss of parental virus. Internal primers for the overlapping FWPV fragments were used for recombinant-specific PCR, together with MVA flanking primers to detect the presence of residual parental virus (the larger recombinant-specific product being product out-competed by any smaller parental-specific product). If parental MVA-LZ virus remained at this stage, the chimeras were subjected to plaque purification. Some chimeras made with 8 kbp FWPV inserts returned PCR products shorter than expected, or no products at all. Such viruses were not further characterised but were labelled as “unstable”.

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Single FWPV gene recombinant MVA, each carrying one gene from the FIR1 locus, were constructed by SOE PCR using single gene primers (012 forward: CATAATACAAGGCACTATGTCCTTTTTCTTCCGAACCTACC, 012 reverse: GTATTCTGGAGGCTGCATCCTTATTAAACCGGAAATGTG, 013 forward: CATAATACAAGGCACTATGTCCAACGCATTTTTACCGTATTG, 013 reverse: GTATTCTGGAGGCTGCATCCACATCAGCAGTTTTATTTTTG). Construction of knock-out and knock-in recombinant FWPV

To construct knock-out FWPV, SOE PCR (using high fidelity Taq polymerase) was used to assemble linear recombination templates from three constituent parts: approximately 350 bp PCR fragments of the FWPV genome from either side, (i) and (ii), of the centre of the target gene, disrupted in the middle (iii) by a VACV p7.5 promoter upstream of the *E. coli gpt* gene. The templates were purified by agarose gel electrophoresis and transfected into CEF previously infected with FWPV FP9. Recovered viruses were bulk passaged three times in CEF under mycophenolic acid selection (in the presence of xanthine and hypoxanthine) for the *gpt* gene then plaque purified. Viral genomic DNA was then extracted and analysed by PCR to confirm disruption of the target gene and loss of parental virus. Primers used were 5′-CGAACGTAGATTCCTACACTC and 5′-TGATAAATTACAACTATTAAC for *fpv012*, 5′-ATTATGAAAACCACGAAAGTC and 5′-AACGTCTATCAGAACCTAAAG for *fpv014*. 
To construct knock-in viruses expressing full length or C-terminally deleted tandem affinity purified (TAP)-tagged *fpv014*, transient dominant plasmids pUC13-FL014TAP and pUC13-CtDel014TAP were constructed in two steps. Firstly the 400bp 3' flanking sequence of *fpv014* was amplified by PCR and cloned into the poxvirus transient dominant TAP vector pUC13TAP (from Prof. G. Smith), such that the 3' flanking sequence was downstream of the TAP tag, generating the intermediate plasmid pUC13TAP014-3'. Subsequently FL or CtDel *fpv014* (minus the stop codon) and 400 bp of upstream sequence were amplified by PCR and cloned into pUC13TAP014-3' so that the *fpv014* ORF was fused in frame with the TAP tag. Constructs were sequenced to ensure no errors had been introduced.

Recombinant TAP-tagged *fpv014* viruses were generated by transfection of the constructs into CEFs infected with a mutant FWPV FP9 previously deleted for the parental *fpv014* by the transient dominant selection method (33) The knock-in mutants were then isolated by the transient dominant selection method under mycophenolic acid selection (in the presence of xanthine and hypoxanthine) for the *gpt* gene. Recombinant viruses were checked by PCR for the presence of the FL or CtDel *fpv014* genes (data not shown).

**Plaque reduction assays**

CEF were pretreated for 16 to 18 h with ChIFN1 diluted in growth medium with 2% NBBS. The medium was aspirated and cells were infected with about 50 plaque-forming units per well. After 90 min incubation, excess virus was aspirated and solid overlay containing 1 % agarose was added to cells. Plaques
were fixed with 10% formaldehyde and stained with 20% crystal violet in 20% ethanol, 30 h (SFV), 3 d (VACV) or 4 d (MVA and FWPV) post-infection, counted and expressed as a percentage of mean (n=3) plaques formed in mock-treated wells. LacZ positive viruses were visualized by addition of solid overlay containing X-gal (0.4 mg ml\(^{-1}\)).

**RNA extraction and processing of samples**

RNA was extracted from cells using an RNeasy kit (Qiagen) according to the manufacturer's instructions. On-column DNA digestion was performed using RNase-free DNase (Qiagen) to remove contaminating genomic DNA. RNA samples were quantified using a Nanodrop Spectrophotometer (Thermo Scientific) and checked for quality using a 2100 Bioanalyzer (Agilent Technologies). All RNA samples had an RNA integrity number (RIN) ≥ 9.6.

**Reverse transcription (RT) PCR and quantitative real-time RT PCR**

qRT-PCR was performed using MESA GREEN qPCR MasterMix Plus for SYBR® Assay dTTP (Euromgitec) according to the manufacturer's instructions. A final volume of 10μl per reaction was used, with 1μl cDNA diluted 1:10 in nuclease-free H₂O as a template. Primers were used at a final concentration of 300nM. qPCR was performed on an ABI-7900HT Fast Real-Time PCR System (Applied Biosystems) using the following programme: 95°C for 5 minutes; 40 cycles of 95°C for 15 seconds, 57°C for 20 seconds, 72°C for 20 seconds; 95°C for 15 seconds; and 60°C for 15 seconds.
The qRT-PCR primer pairs (GGCACTGTCAAGGCTGAGAA and TGCATCTGCCCATT TGATGT for ChGAPDH; TGCTCTCCAGGGGTGCGTTACC and TAGCGCCGTGTCCAACAGCG for fpv014; GTGTTTCAGCCAAAGTAG and AGTAGGTCTTCGTACAGATG for fpv100; ACCTCAAAACAACCTCAG and GTTAATACTTGACTGCTG for fpv168) were validated by generating standard curves using PCR products corresponding to each gene. A 10-fold dilution series was made for each PCR product and 1 µl was used with the MESA green qPCR mastermix. Ct values were analysed using SDS2.3 (Applied Biosystems). The slopes of the standard curves were used to identify the amplification efficiencies (E) of the qRT-PCR primer pairs, using the equation E = 10(-1/slope) - 1. Only qRT-PCR primer pairs with efficiencies of 90-110% were used further. The linear correlation coefficient (R2) was used to assess the linearity of the standard curve. Standard curves with an R2 value of >0.985 were used.

Data were analysed using SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems). All target gene expression levels were calculated relative to expression levels of the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which were shown to remain constant over 24 h in uninfected and FP9-infected cells, and the target gene expression level in control CEF, using the comparative Ct method (also referred to as the 2−ΔΔCT method).

**Tandem affinity purification**

FPV014 was cloned into a TAP-tagging vector (kind gift of B. Ferguson) comprising a pcDNA4-TO (Invitrogen) backbone and a C-terminal SF-TAP tag.
(34). CTAP-tagged 014 was transfected into DF1-TR cells and resulting transformants selected using BlasticidinS and Zeocin, generating a CTAP014 inducible cell line. Twenty T175 flasks of CTAP014 were grown up and expression of 014 was induced in ten of them by the addition of doxycycline. Twenty-four hours following induction cell lysates were made and sequential immunoprecipitations carried out, firstly with Streptactin beads (IBA) and secondly with Flag beads (Sigma). Final elutions were concentrated using a 3K MWCO micron ultrafiltration device (Millipore) and samples were run on an SDS polyacrylamide gel and stained using coomassie blue. Protein bands that appeared in the induced and not the uninduced sample were excised and submitted for tandem mass spectrometry at the CISBIO mass spectrometry core facility (Imperial College London, managed by Dr Paul Hitchen).

RESULTS

Fowlpox virus is resistant to the antiviral effects of chicken type I IFN.

The sensitivity of FWPV FP9 replication to exogenous ChIFN was measured by plaque reduction on primary chicken embryo fibroblasts (CEF) in the presence of recombinant ChIFN1. In our preliminary studies in CEF, the IC50 for the control virus, Semliki Forest virus (SFV) A7(74), was by definition, 1 U ml\(^{-1}\), while that for VACV WR (VTN) was 176 U ml\(^{-1}\) (Fig. 1). VACV MVA, which was derived by extensive CEF passages (more than 500), during which it underwent considerable deletion of genomic sequences relative to its parental strain, chorioallantois Vaccinia virus Ankara (35-37), had an IC50 of only 7 U ml\(^{-1}\). FP9,
however, proved far more resistant (Fig. 1), such that the number of plaques was reduced only by 36% at the highest concentration of ChIFN used (1563 U ml⁻¹).

The ChIFN used in this preliminary study caused some cytotoxicity at high concentrations (Fig. 1A), so the IC₅₀ for FP9 could initially only be estimated upon extrapolation by non-linear regression, yielding a tentative figure of 2570 U ml⁻¹.

**Identification of FWPV chimeric MVA with enhanced resistance to ChIFN1.**

The observation that MVA is almost 400-fold more sensitive to the antiviral effects of ChIFN1 than FP9 (Fig. 1) presented the possibility of a gain-of-function genetic screen of the FWPV genome, using MVA as a recipient, to attempt to identify gene(s) involved in the enhanced resistance of FP9 to ChIFN1.

A panel of 63 MVA-FWPV chimeras were generated, as described in Materials and Methods, together spanning much of the FP9 genome in 4 or 8 kbp inserts (Fig. 2a), inserted close to deletion II of MVA (35), in an intergenic region mid way between the end of MVA023L and the start of MVA021L. The design strategy foresaw a tiling path of 8 kbp fragments overlapping by 4 kbp but not all chimeras were isolated and some (which we define as “unstable”) proved to have smaller inserts than expected, as determined by PCR analysis, possibly due to competition between equivalent VACV and FWPV proteins. The library was evaluated to cover 85% of the FWPV ORFs (with 64% of the ORFs in one or more viruses).
Although poxvirus promoters from VACV have been shown to function in cells infected by FWPV, and *vice versa*, the ability of an MVA chimera to express a FWPV protein from its cognate promoter in the inserted FWPV genomic DNA was demonstrated by western blotting, with monoclonal antibody (MAb) DE9, cell-free extract from cells infected with chimeras. MAb DE9 recognises the 63 kDa FWPV equivalent of VACV p4c, encoded by fpv191 (38), located in fragment 50 of the chimeric library. Three independent chimeras (MVA-f50a, b and c) were isolated following separate transfections with fragment 50. Chimeras MVA-f50a and MVA-f50c but not “unstable” chimera MVA-f50b nor control chimera MVA-fC2b showed strong expression of fpv191 (Fig. 2b).

A total of 28 of the chimeras (none of which were classified as “unstable”) were screened, by plaque reduction assay, for enhanced resistance to low titres of ChIFN1 (10 to 16 U ml$^{-1}$). Data from the screening of 18 such chimeras are shown in Fig. 3. Chimera MVA-f3La was readily identified as having significantly higher resistance than the control and other chimeras, whether screened as third bulk-passage (Fig. 3a) or plaque-purified (Fig. 3b) virus. Only one other chimera, MVA-f6b, demonstrated resistance to ChIFN1 significantly higher than the control (but less than that of MVA-f3La; data not shown). It carried an 8kbp locus that we named Fowlpox virus IFN Regulator 2 (FIR2), comprising position 23791 to 32167 of the FWPV FP9 sequence accession AJ581527 (25) and spanning ORFs fpv022 to fpv026, inclusive, all of which are transcribed from right to left. Chimera MVA-f3La carried only a 4 kbp segment of the FWPV genome (position 11900 to 16043 of the FP9 sequence), spanning three ORFs (fpv012 to
We named this genomic locus FIR1.

Identification of an FWPV gene contributing to virus resistance to ChIFN1.

Firstly, expression of FWPV genes fpv012 and fpv014 in CEF infected with MVA-f3La but not with control MVA-fC2a was demonstrated by qRT-PCR; in fact both FWPV genes were expressed at higher levels from the chimera than from donor FP9 (Fig. 4). Then recombinant MVA, each carrying just a single gene from the FIR1 locus, were constructed by SOE PCR. Expression of fpv014, as determined by qRT-PCR, was lower in MVA-F.014 (13 & 60 fold, relative to FP9, at 8 & 16 hpi, respectively) than in MVA-f3La (26 & 81 fold), while fpv012 expression was higher in MVA-F.012 (15 & 43 fold) than in MVA-f3La (5 & 20 fold) (Fig. 4).

The single gene recombinant MVA were similarly screened for enhanced resistance to ChIFN1 by plaque reduction assay. Preliminary experiments (for example Fig. 5a) showed that fpv013 did not contribute to enhanced ChIFN1 resistance. Subsequent experiments demonstrated that plaque formation by MVA-f3La, under different concentrations of ChIFN1, was significantly higher than that by the control MVA-fC2a and the recombinant (MVA-F.012) expressing only fpv012 but not the recombinant (MVA-F.014) expressing only fpv014 (Fig. 5b and c). MVA-F.014 consistently plaqued at considerably higher efficiency than MVA-fC2a and MVA-F.012 but the results did not reach statistical significance. The IC50 of ChIFN1 for each of the viruses was therefore derived in a further experiment. The IC50 for MVA-f3La (68 U ml⁻¹) was significantly higher than those for MVA-fC2a (25 U ml⁻¹) and MVA-F.012 (18 U ml⁻¹). Although that for
MVA-F.014 (41 U ml$^{-1}$) was not significantly higher than that for MVA-fC2a, it was significantly higher than that for MVA-F.012 (Fig. 5d). It is therefore only $fpv014$ that consistently shows a contribution towards the resistance to ChIFN1 observed in MVA-3La.

Expression of $fpv014$ by FWPV.

Analysis of $fpv014$ mRNA expression by qRT-PCR revealed that its levels and kinetics in CEF infected with parental FWPV FP9 are comparable to those of $fpv100$ (Fig. 6a), with expression clearly detectable at 2h (in contrast to late gene $fpv168$) and increasing to 8h in an AraC-sensitive manner (though in contrast to the highly expressed late gene $fpv168$, low level expression is still detectable in the presence of AraC). Gene $fpv100$ is an ortholog of VACV $E4L$ (VACWR060), which encodes RNA polymerase subunit RPO30 and shows group E1.1 expression kinetics (39).

Antibodies for $fpv014$ are still unavailable but a TAP-tagged version of the gene was inserted back into the native locus under control of the cognate promoter and its expression was demonstrated by western blotting of lysates with anti-FLAG antibodies (Fig. 6b).

Characterization of an $fpv014$ knockout mutant of FWPV.

In an accompanying paper (Laidlaw et al.; JVI02736-12), a library of single gene knockout FWPV was screened to identify genes involved in blocking the induction of chicken IFN-β (ChIFN2 (31)). Another gene in the FIR1 locus, $fpv012$, was shown to contribute towards blocking the induction of ChIFN2.
mediated by transfected dsRNA analog, poly(I:C). Conversely, even though
fpv014 contributed to the enhanced resistance to the established antiviral state
observed for chimeric MVA-f3La carrying the FIR1 locus, it did not contribute
towards blocking the transfected poly(I:C)-mediated induction of ChIFN2 in
immortalized chicken fibroblast cell line DF-1 cells (Fig. 7A). Nor did fpv014,
unlike fpv012, inhibit the transfected poly(I:C)-mediated induction of ChIFN2
when expressed ectopically from a eukaryotic promoter (data not shown). As
fpv014 conferred enhanced resistance to MVA chimeras, then its loss from FWPV
FP9 should result in decreased resistance of the knockout virus. The IC50 of
ChIFN1 for the fpv014-knockout virus was therefore derived in comparison with
that for parental FP9 (Fig. 7B) using ChIFN1 that did not cause the cytotoxicity
seen in the preliminary study (Fig. 1A), so that the IC50 did not have to be
calculated by extrapolation. At 2008 U ml⁻¹, the IC50 for the fpv014-knockout
virus proved to be significantly different to that for parental FP9 (5564 U ml⁻¹).

**IFN modulator** fpv014 **is a member of the ANK/PRANC poxvirus gene
family.**

The inhibitor of IFN responses identified by this study, fpv014, is encoded by a
member of the largest poxvirus gene family, These proteins have an N-terminal
domain (INTERPRO IPR020683) containing multiple copies (7 in the case of
fpv014) of the ankyrin repeat (ANK; INTERPRO IPR002110). Most of them,
including fpv014 (Fig. 8a), have been described by Mercer and colleagues (40) as
ANK/PRANC proteins, with multiple ANKs and a C-terminal F-box like motif in a
PRANC domain (Pox protein Repeats of ANkyrin – C terminal; INTERPRO
IPR018272).
fpv014 interacts with proteins of the SCF complex

To identify ligands for fpv014, its gene was cloned into a C-terminal TAP tagging vector placing its expression under the control of the tetracycline operator element. Stable transformants containing the TAP-tagged constructs were established in a derivative (S. Laidlaw, unpublished) of the immortalised DF-1 chicken fibroblast cell line expressing a tetracycline-dependent transactivator. Tandem affinity purification was then conducted on lysates from non-induced and doxycycline-induced cells. Tandem mass spectrometry of co-precipitated bands purified by SDS-PAGE demonstrated that fpv014 is complexed with chicken Cullin-1 (Fig. 8b). In this regard, fpv014 behaves like several other members of the ANK/PRANC family from poxviruses that infect mammals, namely cowpox virus, ectromelia virus, myxoma virus, Orf virus and vaccinia MVA (41-46), indicating that fpv014 also functions via the SCF ubiquitin ligase complex. The PRANC proteins interact indirectly with cullin-1 via Skp1 as an adaptor. Skp1 was not identified in any of the bands excised for mass spectrometry so the affinity-purified TAP-tagged fpv014 eluate (and fpv014 non-expressing control cell lysate) were analysed by western blotting. Non-reducing conditions were used for SDS-PAGE, as the samples had been prepared for compatibility with subsequent mass spectrometry. Under these non-reducing conditions, TAP-tagged fpv014, detected in the purified eluates from expressing cell lines (but not in control lysates) by antibody recognising the FLAG epitope in the TAP tag, migrated as two pairs of bands (the upper of each pair being predominant; Fig. 8c). The lower pair migrated just above the 50 kDa marker, consistent with the expected size (53 kDa) of TAP-tagged fpv014. The upper pair...
migrated just below the 75 kDa marker, consistent with a 72 kDa complex between TAP-tagged fpv014 and Skp1 (19 kDa). Immunoblotting detected Skp1 in the control lysates, at the expected size of 19 kDa (Fig. 8c). In the purified eluates from TAP-fpv014 expressing cells, some Skp1 was evident at 19 kDa, but most of it co-migrated (at 72 kDa) with the slower migrating pair of TAP-tagged fpv014 bands (Fig. 8c). Immunoblotting also detected cullin-1 in the control lysates, at the expected size of 90 kDa (Fig. 8c). The cullin-1 in the purified eluates from TAP-fpv014 expressing cells, however, migrated slightly slower than in the control lysates (consistent with the 98 kDa ubiquitinated form of Cullin-1 observed to interact with the myxoma virus PRANC protein MNF (41)). Even though this cullin-1 co-purified with TAP-tagged fpv014 in the eluate, it was not observed in a complex with TAP-tagged fpv014 (predicted 143 kDa, or 162 kDa with the addition of Skp1) on the immunoblot, whether due to SDS-sensitivity of the complex or to the inability of the antibody to bind complexed cullin-1. We have been unable to source an antibody for another member of the complex, RBX/Roc1, that functions appropriately with chicken cell extracts.

**DISCUSSION**

Vaccinia virus (VACV), the type species of the orthopoxviruses and the most extensively studied poxvirus, has been shown to encode a considerable number of proteins that, though non-essential for replication *in vitro*, contribute to virulence and/or the ability of the virus to replicate *in vivo*. Such proteins generally modulate the innate immune system of the host, and are generically known as immunomodulators. Initially, many of these proteins were identified by their sequence similarity with host immune effectors, probably derived by...
lateral gene transfer. Examples include soluble binding proteins for IFNs and interleukins (6, 7, 47, 48). Similar proteins are encoded by members of other genera of mammalian poxviruses (49, 50). Later, such proteins were identified by functional or ligand-binding studies (51, 52) and, more recently, by structural (but not primary sequence) similarity to host effectors (14, 16).

Such approaches have not proved particularly successful for identifying immunomodulators encoded by avipoxviruses (10, 12, 23). Despite the apparent absence from FWPV of IFN modulators equivalent to those found in VACV, and of other candidate IFN modulators readily identified by their sequence homology with known host components or targets of the IFN system, our data show that FWPV effectively subverts the antiviral effects of avian IFN, even in an established antiviral state. Indeed, FWPV can even rescue co-infecting IFN-sensitive viruses such as SFV (unpublished data), indicating that at least some of the mechanisms can operate in trans.

A two-stage genetic screening strategy was employed in an attempt to identify FWPV genes involved in resisting the antiviral effectors of the type I IFN system. The first-stage screen identified a locus comprising just 3 genes (*fpv012, fpv013 and fpv014*) and the second stage identified *fpv014*. The first-stage chimera, MVA-f3La, carrying 3 genes, appeared more resistant than the one carrying the only gene that contributed to ChIFN1 resistance (*fpv014*) but qRT-PCR analysis later revealed that *fpv014* expression levels were lower in the single gene chimera than in the 3 gene chimera, suggesting that the levels of *fpv14* expression account for the difference in the levels of resistance observed. It is
notable that the fold change in IC50 of the MVA chimeras upon introduction of

$fpv014$ is comparable in scale (but opposite in direction) to the fold change in

FWPV FP9 upon deletion of $fpv014$, reinforcing the view that the MVA screen

offers a valid representation of the role of FWPV IFN modulators in their normal

context.

Both $fpv014$ and $fpv012$ are members of an extensive gene family encoding ANK

proteins, which are found throughout the evolutionary tree in more than 40,000

proteins from viruses and bacteria to higher plants and animals. They mediate

protein-protein interactions and so are found in proteins with all manner of

functions, roles and localisation (53). With the curious exception of MOCV,
mammalian poxviruses all encode ANK proteins, the actual number varying from

4 to 31 members (54, 55) but the higher numbers often represent partial

pseudogenes and the actual number of orthologous groups in mammalian

poxviruses is estimated as being from 5 in parapoxviruses to 15 in

orthopoxviruses (55). The family appears to have expanded considerably in the

avipoxviruses, with 31 members in a pathogenic FWPV (10) and 51 in a

pathogenic 1948 isolate of CNPV (11). The number of orthologous groups cannot

yet be estimated for the avipoxviruses, because so far only the genome

sequences of FWPV and CNPV have been published, but the known ANK genes

represent 11% and 15%, respectively, of the total gene complement of these

viruses. About 70% of them have F-boxes suggesting they are likely to be intact,
in contrast with only 36% in the only mammalian poxvirus that encodes more

than 20 ANKs, horsepox virus (55, 56). Extensive passage (more than 430 times)
of pathogenic FWPV HP-1 through CEF culture by Anton Mayr, with concomitant
attenuation (57), led to the loss or disruption of 12 FWPV ANK genes in FP9 but the only change to fpv014 resulted in a conservative Asp to Glu substitution at residue 225 of fpv014 (25). Given the high level of resistance of FP9 to ChIFN1, we have no reason to expect that HP-1 fpv014 should be any more effective than that of FP9. Apart from FWPV, the only other member of the Avipoxvirus genus for which the genome sequence has been published is CNPV, which is considerably diverged from FWPV, displays significant differences in gene complement (10, 11) and is found in a different major clade of the genus (58). Comparisons between ANK proteins can be problematic but Sonnberg et al. (55) reported the most likely CNPV ortholog of fpv014 to be cnpv019 (48% amino acid identity). These orthologs are not in syntenic positions; and we have not yet assessed whether cnpv019 can contribute to enhanced ChIFN1 resistance in the MVA model.

The combination of N-terminal ANK and C-terminal F-box/PRANC domains found in fpv014 is essentially restricted to poxviruses, though ANK/PRANC genes have now been found in Nasonia parasitic wasps, apparently transferred to them horizontally via endosymbiotic Wolbachia rickettsial bacteria (59). The PRANC domains of a number of mammalian poxvirus ANK proteins have been shown to mediate interaction with core components of the ubiquitin ligase complex (41, 42, 45, 46, 60) It was postulated (40, 43) that such interactions would target for ubiquitination ligands captured by the N-terminal ANK domains. However, the identities of cellular ligands captured by the ANK domain have been established for only an extremely small minority of poxvirus ANK proteins (61). Thus Akt appears to be the target for myxoma virus MT-5 (62),
while NF-κB is bound by variola virus G1R (63) and its cowpox virus (CPXV) ortholog CPXV006 (64) as well as by CPXV CP77/CPXV025 (42).

Despite considerable effort, we have been unable to identify a cellular ligand for the N-terminal ANK domain of fpv014. Although it was not our main goal, we were able to demonstrate tandem affinity co-purification of chicken cullin-1 (by mass spectrometry and immunoblotting) and chicken Skp1 (by immunoblotting only) with TAP-tagged fpv014. This indicated that fpv014 interacts with the avian ubiquitin ligase complex (presumably via the F-box) in the same way that PRANC proteins of mammalian poxviruses have been shown to interact with the mammalian complexes. The immunoblotting was performed after non-reducing SDS-PAGE, not to look for complexes but for compatibility with subsequent mass spectrometry. The observation that most of the co-purified Skp1 co-migrated with a substantial proportion of the TAP-tagged fpv014 was, therefore, somewhat serendipitous. However, it suggests that fpv014 and chicken Skp1 can form an SDS-stable complex. Unfortunately, we were not able to further investigate whether the complex was reductant-sensitive. We are not aware of reports of similar F-box protein:Skp1 gel complexes (though such complexes are observed elsewhere (65, 66)), nor is it apparent to us that others in the field have looked directly for such a complex, or have been in a position to observe it. The cullin-1 that co-purified with TAP-tagged fpv014, however, does not appear to form a co-migrating complex with it on the immunoblot. Because the interaction of cullin-1 with F-box proteins is indirect, via Skp1, it is not perhaps not surprising that an fpv014:cullin-1 complex should be less stable than an fpv014:Skp1 complex. Although the cullin-1 that co-purified with TAP-tagged
fpv014 did not form a complex with the fpv014 on the immunoblot, it migrated more slowly than the cullin-1 in the control lysate. There is a precedent for this observation in that two similar size bands co-purified with GFP-tagged myxoma virus MNF protein. The predominant slower form immunoblotted with an unspecified anti-ubiquitin antibody (41). There is no indication at this stage whether fpv014 stabilised the modified form of cullin-1 in the cell, or whether it was solely (or predominantly) the modified form that co-purified with TAP-tagged fpv014. We have not been able to investigate whether the cullin-1 that co-purified with TAP-tagged fpv014 was ubiquitinated, but we are aware that the size difference observed between it and the cullin-1 from control lysates could equally correspond to the well-reported addition of the ubiquitin-like NEDD8 adduct, rather than ubiquitin itself (67-69).

Data shown here (Fig. 7 and unpublished) demonstrate that fpv014 does not inhibit transfected poly(I:C)-mediated induction of the ChIFN2 promoter. There are currently no clues as to the cellular target(s) of fpv014, and gaps in our understanding of the chicken type I IFN system render their identification even more complicated. Although the chicken system is largely equivalent to that of mammals, the as yet incomplete (and incompletely annotated) chicken genome sequence revealed significant differences between the mammalian and chicken systems. The absence of the RIG-I gene from the chicken, but not from all avian species (70), and the retention of mda-5 (71, 72) have already been reported and their possible implications considered. Other key components remain unaccounted for. For instance, two major transcription factors implicated in type I IFN mRNA expression in mammals are constitutively expressed IRF-3 and
IFN-inducible IRF-7 (73), but in the chicken the gene encoding only one of them (ChIRF-3, NP_990703) is found, although it is actually most closely related to mammalian IRF-7. Genes encoding IRF-9 and STAT-2 have also not yet been identified in the chicken (S. Goodbourn and C. Ross, unpublished). The biological consequences of these differences remain unclear but they, combined with a relative dearth of reagents for the chicken system, are likely to complicate elucidation of the mode of action of the FWPV IFN modulators, as well as understanding the interaction of avian innate systems with avian pathogens, not least with important zoonotic agents such as avian influenza virus H5N1 and West Nile virus. However, this new avian virus IFN modulator, together with another (fpv012, which blocks dsRNA-mediated induction of the ChIFN2 promoter) reported in an accompanying manuscript, will provide useful reagents for probing the specific nature of the avian innate defences.

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FIGURES LEGENDS

Figure 1. Sensitivity of viruses to recombinant ChIFN1. Plaque reduction assays were performed using CEF infected with Semliki Forest virus, FWPV FP9 or vaccinia virus (VACV) VTN or MVA in the presence of recombinant ChIFN1. (A) Photograph of crystal violet-stained CEF showing titration of ChIFN1 inhibition of FP9 and MVA plaque formation. (B) Graph showing plaque production as a function of ChIFN1 concentration. The dotted line indicates the 50% reduction end-point. Plaque formation is expressed as a percentage of plaques formed in mock-treated wells (mean of n=3). Each point represents an individual well, except for FP9 where points are the mean (4 wells from 2 independent experiments) ± 1 SD. Symbols: squares, FP9; circles, VACV VTN; closed triangles, MVA II/85; open triangles, SFV A7(74).
Figure 2. Characterization of the FWPV chimeric MVA library. (A) Distribution on the FWPV FP9 genome of fragments inserted in the chimeric MVA library. The integrity of FWPV inserts was examined by PCR, using internal primers from the overlapping fragments with primers flanking the insertion site. Inserts that were shorter than expected were labeled as “unstable”. Where duplicate viruses containing a particular fragment were recovered, the fragment is classified here as a "Full-length insert" if at least one derived recombinant virus was of the anticipated size. Regions of the FP9 genome that were not present in the recombinant MVA library are highlighted as "uncloned genome".

To scale. (B) Expression of fpv191 by MVA chimeras MVA-f50a and MVA-f50c. Lysates of uninfected or infected CEF were subject to 10% SDS-PAGE then immunoblotted using primary monoclonal antibody DE9 (38) at 1 in 200 and secondary goat anti-mouse IgG (Sigma-Aldrich) at 1 in 25000. Lane 1: Uninfected CEF. Lane 2: FP9 infected CEF. Lane 3: MVA-LZ CEF. Lane 4: MVA-f50a infected CEF. Lane 5: MVA-f50b infected CEF. Lane 6: MVA-f50c infected CEF. Lane 7: MVA-fC2b infected CEF. Amount of cell lysate loaded is expressed relative to that for the FP9-infected lysate (lane 2).
Figure 3. Screening FWPV chimeric MVA for enhanced resistance to ChIFN1.

CEF were pre-treated in triplicate with ChIFN1 (10 U ml⁻¹) or mock treated with DMEM 2% FBS, for 18 h. The medium was aspirated and cells were infected with chimeric viruses at 100 to 300 (A) or 50 to 200 (B) pfu/well. Virus was aspirated after 90 mins and semi-solid overlay was added. A second overlay containing X-gal was added 3 days p.i. Plaque formation for each virus in ChIFN1-treated wells is expressed as the mean (n=3, ± SEM) percentage relative to mock-treated wells. Separate experiments are shown in (A) and (B). The screen shown in (A) used third bulk-passage, mycophenolic acid selected MVA-f3La (subsequently found to contain residual MVA-LZ) but that shown in (B) used fourth passage, plaque-purified MVA-f3La (found to be free of parental MVA-LZ).
Figure 4. qRT-PCR analysis of the expression of FWPV insert-specific genes by first and second stage FWPV chimeric MVA. Expression of mRNA specific for *fpv012* and *fpv014* in FP9- and control (MVA-fC2a) or FWPV chimeric MVA-infected CEF was assayed by qRT-PCR, normalized against that for ChGAPDH. MVA-f3La is a first stage chimera carrying the FIR1 locus, MVA-F.012 and MVA-F.014 are second round recombinants carrying only *fpv012* and *fpv014*, respectively. Expression of mRNA specific for MVA A12L and the FWPV equivalent of A12L (*fpv176*) in FP9- and chimeric MVA-infected CEF, as vector-specific controls, was assayed by qRT-PCR, normalized against that for ChGAPDH. (A) *fpv012*, open bars; (B) *fpv014*, chequered bars; (C) *fpv176*, horizontal hatched bars; (D) A12L, vertically hatched bars. Mean and standard deviation, at 8 and 16 hours post infection (hpi), are plotted from three independent experiments. Note differences in y-axis scales.
Figure 5. Screening and characterization of second-round chimeric MVA to identify the FIR1 gene responsible for enhanced resistance to ChIFN1. CEF cells (in 9 cm² dishes) were incubated with 1 ml DMEM 2% FBS (+ or – ChIFN1 at 27 (A), 13 (B) or 6 (C) U ml⁻¹) for 18 h. The medium was aspirated and cells were infected at 90 to 180 pfu/well. All viruses except control MVA-fC2a were plaque-purified. MVA-f3La is a first stage chimera carrying the FIR1 locus; MVA-F.012, MVA-F.013 and MVA-F.014 are second round recombinants carrying only fpv012, fpv013 and fpv014, respectively. Except in (A), which is an example of several experiments, the numbers of plaques formed in the ChIFN1-treated wells were expressed as a percentage of the mean (n=3) number of plaques formed in mock-treated wells. (D) Plaque reduction assays were performed on FWPV chimeric MVA at a range of ChIFN concentrations from 5 to 200 U ml⁻¹. IC50 values for ChIFN1 with each of the viruses were derived in PRISM (Graphpad) using non-linear fits of the log converted ChIFN1 concentrations against percentage normalized plaque responses. IC50 values are plotted with 95% confidence intervals. Two-way ANOVA: * p<0.05; ** p<0.01.
Figure 6. Characterization of the expression of fpv014 in FWPV. (A) Analysis of the kinetics of expression of mRNA specific for fpv014 in wild-type FWPV was performed by qRT-PCR, using as controls FWPV genes fpv100 (ortholog of VACV E4L; RNA polymerase subunit RPO30) and fpv168 (ortholog of VACV A4L). Expression in the absence and presence of poxviral DNA replication inhibitor AraC was normalized against that for ChGAPDH. Mean and standard deviation are plotted from three independent experiments. (B) Expression of TAP-tagged fpv014, either full-length (FL) or C-terminally deleted (CtDel), inserted back into the native locus in FWPV FP9 under control of its cognate promoter, detected by immunoblotting of SDS polyacrylamide gels with anti-FLAG antibody (Sigma) and anti-mouse secondary antibody (LICOR) as per the manufacturers’ protocols. The immunoblots were imaged using a LICOR Odyssey. Samples were obtained 24 hours post infection at an moi of 3. Molecular weight markers (M) are shown, as are the predicted masses of FL (53 kDa) and CtDel (50 kDa) TAP-tagged fpv014.
Figure 7. Characterization of an fpv014 knockout mutant of FWPV. (A) Like parental FWPV FP9, but unlike one defective in fpv012 (Del012), an FP9 mutant defective in fpv014 (Del014) retains full ability to block poly(I:C)-mediated induction of the ChIFN-2 promoter. Chicken DF1 cells were transfected with the ChIFN-2 promoter reporter (pChIFN-2lucter) and the constitutive LacZ reporter plasmid pJATlacZ. Following recovery for 24 hours, cells were either left uninfected or infected with parental FWPV FP9 or single-gene mutants of FP9, at an MOI of 10. Following infection for 4 hours, cells were either left untreated or were transfected with poly(I:C) (10µg ml⁻¹) and incubated for 16 hours. Luciferase assays were carried out and data were normalised using β-galactosidase measurements. Each sample was compared to the uninfected, poly(I:C)-treated control to calculate percent induction. Results show the mean (n=3) + SD. (B) Plaque reduction assays were performed on parental and fpv014 knockout (Del014) FWPV FP9 at a range of ChIFN1 concentrations from 1 to 10,000 U ml⁻¹. IC50 values for ChIFN1 with each of the viruses were derived in PRISM (Graphpad) using non-linear fits of the log converted ChIFN1 concentrations against percentage normalized plaque responses. IC50 values are plotted with 95% confidence intervals.
Figure 8. *fpv014* is an ANK/PRANC protein that associates with Skp1 and cullin-1.

(A) Domain structure of *fpv014* showing N-terminal ankyrin (ANK) repeats as well as the C-terminal F-box motif and the larger, encompassing PRANC domain. To scale. (B) Chicken cullin-1 amino acid sequence (XP_418878) aligned (dots indicate identical residues) with that of human cullin-1 (NP_003583), showing peptides (underlined) identified by mass spectrometric sequence analysis of proteins co-purified with TAP-tagged *fpv014*. (C) Immunoblot detection of Skp1 and cullin-1 co-affinity purified with TAP-tagged *fpv014*. DF1 control cell lysate ('Control lysate') or final eluate of TAP-affinity purified lysate from a DF-1 cell line inducibly expressing TAP-tagged *fpv014* ('TAP-affinity-purified eluate') were subject to non-reducing 15% SDS PAGE then immunoblotted using mouse anti-FLAG (Sigma Aldrich), mouse anti-Skp1 or mouse anti-cullin-1 (BD Transduction Laboratories) at 1 in 1000 followed by goat anti-mouse IgG (Sigma) at 1 in 25000. Molecular weight markers (M) are shown, as are the predicted masses of TAP-tagged *fpv014* (53 kDa), ChSkp1 (1 kDa) and ChCullin-1 (90 kDa).
A

[ChIFN1] (U ml$^{-1}$)

0 15625 1563 156 16 2

MVA

FP9

B

% plaque formation

[ChIFN1] (U ml$^{-1}$)
Gene expression compared to GAPDH

Time (hours post-infection)

A

B

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<tr>
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<th>CEF</th>
<th>DF1</th>
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<td>FL-TAP014</td>
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<td>FL-TAP014</td>
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% Poly(I:C)-induced ChIFN2luc expression relative to uninfected

Virus
Uninfected
Parental FP9
Del012
Del014

ChIFN1 IC50 (U/ml)

FP9
De1014
### A

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### B

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- **Gallus**: QARGAVPFSPSKGQTPGQAQVGGCLEYKREFLKNLYLNTNLKDGQLDOSVLKFKYT

### C

- **Human**: NAVLKLIEKEKGETINTRLISGVQSYVVELGXNEEDAFKQPTLTVYKRESPOFADT
- **Gallus**: EFPYRESTEFLQOPVTEYMKKAARLEQKRRVWLLHESQDHELARKEQVLIEKHL

- **Human**: EFPYRESTEFLQOPVTEYMKKAARLEQKRRVWLLHESQDHELARKEQVLIEKHL
- **Gallus**: LLARYCSSLKKSSKNEEAELDNLGVNVFKEQKFDKAFKAKLAKKLHHNSA

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