An Interferon-γ-binding Protein of Novel Structure Encoded by the Fowlpox Virus

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Poxviruses have evolved various strategies to counteract the host immune response, one of which is based on the expression of soluble cytokine receptors. Using various biological assays, we detected a chicken interferon-γ (chIFN-γ)-neutralizing activity in supernatants of fowlpox virus (FPV)-infected cells that could be destroyed by trypsin treatment. Secreted viral proteins were purified by affinity chromatography using matrix-immobilized chIFN-γ, followed by two-dimensional gel electrophoresis. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis indicated that the viral IFN-γ-binding protein in question was encoded by the FPV gene 016. The chicken IFN-γ-binding and neutralizing activity of the recombinant FPV016 protein was confirmed using supernatants of cells infected with a recombinant vaccinia virus that lacked its own IFN-γ-binding protein but instead expressed the FPV016 gene. The FPV016 gene product also neutralized the activity of duck and human IFN-γ but failed to neutralize the activity of mouse and rat IFN-γ. Unlike previously known cellular and poxviral IFN-γ receptors, which all contain fibronectin type III domains, the IFN-γ-binding protein of FPV contains an immunoglobulin domain. Remarkably, it exhibits no significant homology to any known viral or cellular protein. Because IFN-γ receptors of birds have not yet been characterized at the molecular level, the possibility remains that FPV016 represents a hijacked chicken gene and that avian and mammalian IFN-γ receptors have fundamentally different primary structures.

Poxviruses have multiple evasion strategies to counteract the antiviral host defense. They code for a number of gene products that are not essential for viral replication. Many of these viral proteins are directed against the host immune system. They either block the complement system (1, 2), interfere with cytokine and chemokine function (3–5), inhibit antigen presentation (6), or influence inflammatory processes (7, 8). A smart poxviral strategy to evade the antiviral defense is to encode soluble proteins that prevent the binding of cytokines to their cognate cellular receptors. Poxviral cytokine-binding proteins with specificity for mammalian tumor necrosis factor-α and -β (9–12), interleukin-1β (IL-1β) (13–15), IL-2 (16), IL-18 (17–19), interferon (IFN)-α/β (20, 21), IFN-γ (22, 23), granulocyte macrophage colony-stimulating factor (16), and various chemokines (24–26) were identified. The importance of IFN-γ in host defense against poxvirus infections was clearly demonstrated in both tissue culture and animal model systems (27, 28). The central role of IFN-γ in the host defense against poxviruses probably explains why this cytokine is one of the main targets of the poxviral immune evasion strategy. In fact, not only does vaccinia virus code for a soluble IFN-γ-binding protein, but it also targets IFN-γ by a virus-encoded phosphatase that interferes with cytokine signaling (29).

A gene encoding a soluble IFN-γ-binding protein was first identified in the myxoma virus genome (22). Homologous genes were subsequently identified in the vaccinia virus (23, 30), the variola virus (31, 32), the swinepox virus (33), the shope fibroma virus (34), and the ectromelia virus (35). Proteins with IFN-γ-binding activity were further detected in supernatants of cowpox virus- and camelpox virus-infected cells (23). Cells infected with tanapox virus were reported to secrete a cytokine-binding protein of unknown structure that can bind IFN-γ, IL-2, and IL-5 (36). Soluble IFN-γ-binding proteins of various poxviruses differ in their specificity for IFN-γ from different animal species. For example, the myxoma virus M-T7 protein preferentially binds and neutralizes rabbit IFN-γ. On the other hand, the IFN-γ-binding proteins of the vaccinia virus, the cowpox virus, and the camelpox virus (23) have broad specificity. They recognize human, bovine, and rat as well as rabbit IFN-γ. The IFN-γ-binding protein of the vaccinia virus further recognizes and neutralizes chicken IFN-γ (chIFN-γ) (37). The mostly broad species specificity of poxviral IFN-γ-binding proteins contrasts with the situation for cellular IFN-γ receptors, which typically show high affinity for IFN-γ from the cognate species only (38). All poxviral IFN-γ-binding proteins described to date show significant homology to mammalian IFN-γ receptors (39). Like their cellular counterparts, they contain two fibronectin type III domains and exhibit features of class II cytokine receptor family members. The poxviral IFN-γ-binding proteins lack transmembrane and cytoplasmic domains.

The fowlpox virus (FPV) belongs to the subfamily chordopoxvirinae. It is the prototype of the genus avipoxivirus. The com-

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††† The abbreviations used are: IL, interleukin; IFN, interferon; chIFN-γ, chicken IFN-γ; FPV, fowlpox virus; DMEM, Dulbecco’s modified Eagle’s medium; CEF, chicken embryo fibroblast; pfu, plaque-forming unit; m.o.i., multiplicity of infection; GBP, guanylate-bind-

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plete genome sequence of a single FPV strain is presently available (40). It contains 260 open reading frames, including that of gene 073, which shows low homology to viral and cellular IL-18 binding proteins (17–19). Surprisingly, sequence analysis failed to provide evidence for FPV genes that might code for IFN-γ-binding proteins.

Here we showed that FPV gene 016 codes for a secreted protein that binds and neutralizes the activity of chIFN-γ. This protein shows no significant homology to known IFN-γ-binding proteins of other poxviruses or to any known cellular IFN-γ receptors.

EXPERIMENTAL PROCEDURES

Cells—The quasi fibroblast cell line CEC-32 and the chicken macrophage cell line HD-11 were grown in Dulbecco’s modified essential medium (DMEM) supplemented with 8% fetal bovine serum and 2% chicken serum. CV-1 cells were grown in DMEM supplemented with 5% fetal bovine serum. Low passage human embryonic fibroblasts and BALB/c mouse embryonic cells were maintained in DMEM supplemented with 10% fetal bovine serum. Primary chicken embryo fibroblasts (CEF) were cultured in DMEM supplemented with 7% newborn calf serum.

Fowlpox Virus—FPV strain HP1-447 was a gift from Dr. A. Mayr (University of Munich, Germany). FPV stocks were grown on primary CEFs. Viral titers were determined by plaque assay on CEFs. Supernatants of FPV-infected cells containing immunomodulatory proteins were produced by infecting CEFs at a multiplicity of infection (m.o.i.) of 0.01 p.f.u./cell. Cell supernatants were harvested 6 days later and stored at −80°C.

Recombinant Vaccinia Viruses—Vaccinia virus strain VVΔBR, which lacks the BBR gene, was a gift from Dr. G. L. Smith (Wright-Fleming Institute, London, Great Britain). Vaccinia virus VVΔBR/FPV016, which expresses the FPV gene 016 under the control of the vaccinia virus p7.5 promoter, was constructed by transient transfection of CEC-32 cells, 10 units/ml of recombinant chIFN-γ produced by transfection monkey COS cells (46) were mixed with various dilutions of supernatant from poxvirus-infected cells and incubated for 1 h at room temperature. CEC-32 cells (2 × 10^6 per well) were then treated at 37°C with 2 ml of the various mixtures for 18 h before RNA isolation. RNA was subjected to Northern blot analysis and hybridized with radiolabeled BP cDNA probes.

Similar assays were employed to determine whether recombinant FPV016 protein can neutralize the IFN-γ of other species. Duck IFN-γ produced in COS cells (47) is a kind gift of Dr. U. Schultz, University of Freiburg, Germany, which is unstable on quail cells (47), was assayed on CEC-32 cells. Human IFN-γ and bovine Molecular Biologicals asayed on low passage human fibroblasts, whereas rat and mouse IFN-γ (R&D Systems, Wiesbaden, Germany) were assayed on low passage BALB/c mouse embryonic cells.

Assay for Neutralization of chIFN-γ-mediated Nitric Oxide Production in Macrophages—To determine the neutralization of chIFN-γ-mediated nitric oxide production, 20 units/ml recombinant chIFN-γ from COS cells were incubated with a supernatant from FPV-infected cells at a 1:4 dilution for 1 h. About 3 × 10^4 HD-11 cells were then seeded into each well of a 96-well microtiter plate before they were treated at 37°C with 100 μl of the various mixtures for 24 h. Nitric oxide production was monitored as a function of nitrite accumulation in the HD-11 cell culture medium using the Griess assay (48, 49).

Assay for Neutralization of IFN-α-mediated Antiviral Activity—To determine whether culture supernatant of VVΔBR/FPV016-infected cells contained an activity that would neutralize chIFN-α, 2-fold dilutions (starting from 200 units/ml) of recombinant cytokine produced in transfected COS cells were incubated with culture supernatants at 1:10 dilution for 1 h and then added to CEFs for 15 h. The cells were then challenged with vesicular stomatitis virus, and virus-induced damage was assessed 24 h later as described (50).

Tryptic Digestion of Proteins in the Supernatant of FPV-infected Cells—Twenty units of trypsin agarsose (Sigma) were incubated with 500 μl of supernatant from FPV-infected cells at 37°C. Incubation was done under constant rotation on the overhead shaker for 15 h. The agarose beads were then removed by centrifugation, and the resulting supernatants were incubated with chIFN-γ and then applied to the indicator cells.

Binding Assay to Demonstrate Direct Interaction of the FPV016 Protein with chIFN-γ—Protein-loaded nickel-agarose beads were prepared as described purification procedures for histidine-tagged proteins in which, however, the final elution step was omitted. Protein-loaded beads were incubated for 15 h at 4°C in 1 ml of buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol, 2 mM imidazole, and proteinase inhibitors) with a 1-ml sample of 35S-labeled supernatant from poxvirus-infected cells. The beads were washed with 1 ml of buffer containing 50 mM imidazole and 1 ml of buffer B (100 mM KCl, 20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 20% glycerol, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol, 20 mM imidazole, and proteinase inhibitors). Bound proteins were eluted by incubation for 10 min at 95°C in 50 μl of SDS gel-loading buffer. They were analyzed by electrophoresis through a SDS-polyacrylamide gel (15%) and visualized by Coomassie Blue staining followed by autoradiography.

Two-dimensional Gel Electrophoresis—For isoelectric focusing, the IPophor system (Amersham Biosciences) was used. Separation of proteins by their molecular weights was performed in a SE600 Hoefer apparatus. Separation of proteins by their isoelectric points was performed in a Mini-PROTEAN II (Bio-Rad) apparatus. Proteins were visualized by silver or Coomassie Blue staining.

RESULTS

Supernatants of FPV-infected Cells Contain Proteins with chIFN-γ-neutalizing Activity—To determine putative FPV-encoded proteins with IFN-γ-binding activity, we tested whether supernatants of FPV-infected CEF would neutralize the chIFN-γ-mediated induction of the GBP gene (46) in quail CEC-32 cells. CEFs were infected for 6 days with FPV strain HP447 (51) before the culture medium was harvested and mixed at various ratios with chIFN-γ for 1 h before the mixtures were added to the CEC-32 indicator cells. RNA was isolated at 16 h post onset of cytokine exposure and subsequently used for Northern blot analysis. As controls, CEC-32
cells were incubated either with medium alone or chIFN-γ alone. Strongly reduced induction of the GBP gene was observed when chIFN-γ was used in combination with the supernatant from FPV-infected cells (Fig. 1A). At a 10^{-2} dilution of the FPV supernatant, the GBP signal was almost completely lost. At a 10^{-3} dilution, the GBP signal was easily detectable, but it was still less intense than that of the positive control. A supernatant from vaccinia virus-infected HeLa cells, known to contain chIFN-γ-neutralizing activity (37), neutralized GBP induction by chIFN-γ with comparable efficacy (Fig. 1A).

chIFN-γ-mediated induction of the NOS-2 gene in chicken macrophages (46) was measured to verify the neutralizing activity of FPV supernatants in an independent second biological test system. For this purpose, chIFN-γ was incubated with supernatants from FPV-infected CEF for 1 h before the samples were added to HD-11 chicken macrophage cells. Nitrite concentration in the culture supernatant was measured 24 h later. As negative and positive controls, the HD-11 cells were incubated with either medium alone or chIFN-γ alone. We found that FPV supernatants potently neutralized the chIFN-γ-induced accumulation of nitrite in the culture medium of HD-11 cells. At a 1:4 dilution, the FPV supernatants blocked this induction nearly completely (Fig. 1B).

To determine whether the observed chIFN-γ-neutralizing activity resulted from the action of one or more FPV proteins or else from non-proteinaceous factors, an aliquot of the supernatant was treated with trypsin that was immobilized on agarose beads. After removal of the trypsin beads by centrifugation, the treated supernatant was incubated at a 1:4 dilution with chIFN-γ for 1 h as above before the NO induction assay was performed. The trypsin-treated supernatant was no longer able to neutralize the activity of chIFN-γ (Fig. 1B), suggesting that viral proteins were responsible for the observed neutralization phenomenon.

**Proteins in Supernatants of FPV-infected Cells That Bind Matrix-immobilized chIFN-γ**—To identify viral proteins with chIFN-γ binding activity, FPV-infected chicken fibroblasts were metabolically labeled with radioactive amino acids for 15 h before the cell supernatant was harvested. Supernatants of uninfected cells that were metabolically labeled for the same period of time served as a negative control. The radiolabeled supernatants were then incubated at 4 °C for 15 h with samples of agarose beads that were loaded with either recombinant histidine-tagged chIFN-γ (His-chIFN-γ) or a control protein (His-MxA). After extensive washing, bound proteins were eluted from the beads and analyzed by SDS-PAGE and autoradiography. Two distinct bands (arrowheads) were observed when labeled supernatant from FPV-infected cells was allowed to react with immobilized chIFN-γ. The gel positions of molecular weight markers are indicated.
proteins for MALDI-TOF MS peptide fingerprint analysis, two-dimensional gel electrophoresis was performed. For this experiment, infected chicken embryo cells were maintained for 14 h in serum-free medium before the culture supernatant was harvested. Proteins were concentrated by ethanol precipitation, and two-dimensional gel analysis was performed using 150 μg of protein per gel. To facilitate the detection of protein spots that might represent the putative soluble IFN-γ-binding factor of FPV, we added small amounts of radiolabeled proteins purified by affinity chromatography using matrix-immobilized chIFN-γ as described above. After electrophoresis in the second dimension, a complex pattern of protein spots was visualized by silver staining (Fig. 3A, left panel). Autoradiography of the dried gel revealed nine spots (Fig. 3A, right panel), namely four prominent spots migrating at about 32–34 kDa and five minor spots migrating at about 38–42 kDa. The two groups of spots appeared like pearls on a string, suggesting that they might represent a single protein with modifications that affect charge and mass. Two of the most prominent spots seen in the autoradiographic picture could be assigned to protein spots of the stained gel (Fig. 3B, arrows). These two proteins were also visible on a Coomassie Blue-stained gel that was run in parallel (data not shown). They were excised and used for MALDI-TOF MS peptide fingerprint analysis.

chIFN-γ Binding Activity is Encoded by the FPV016 Gene—Using Matrix Science’s MASCOT software, we generated a data base with all FPV sequence information available in data bases. Virtual complete and non-complete tryptic digests of every potential FPV gene product were performed, and the masses of all resulting peptide fragments were calculated. The MASCOT software was then used to compare these values to the experimentally derived values. This analysis yielded single hits for both of the protein samples that we had retrieved from our two-dimensional gels. Both proteins corresponded to the predicted product of the FPV016 gene (Mowse Scores of 77 and 71, respectively). The tryptic fragments of the FPV016 protein identified by MALDI-TOF MS analysis are depicted in Fig. 4. The fragment starting at polypeptide position 79 was only found in one of the two gel spots, whereas the other three fragments were present in both samples.

The FPV016 gene codes for a polypeptide with 238 residues. It is located in the left terminal region of the viral genome, which is a typical position for genes encoding immunomodulatory proteins of poxviruses (2). Computer-based sequence analysis using program SignalP (52, 53) identified a potential signal peptide at amino acid positions 1–23, which is followed by a putative signalase cleavage site. Three potential Asn-X-Ser/Thr N-glycosylation sites are present. With help of the program NetOGlyc 2.0 (54), a potential O-glycosylation site was identified that includes Thr at position 93. The program PFAM (55) identified an immunoglobulin domain that includes amino acid residues 29–108. Cysteine residues forming a predicted disulfide bridge are marked by arrowheads in Fig. 4.

Recombinant FPV016 Protein Binds and Neutralizes chIFN-γ—To confirm that the FPV016 gene product indeed can bind to chIFN-γ and neutralize its biological activity, we constructed several plasmids designed to direct polymerase II promoter-driven expression of the FPV016 gene in mammalian or avian host cells. None of these constructs yielded a biologically active protein (data not shown). To determine whether poxviral ex-
pression systems might work better, we constructed a recombinant vaccinia virus that lacks the B8R gene, which encodes a soluble IFN-γ receptor, but instead expresses the FPV016 gene under the control of the vaccinia virus promoter 7.5 (VVΔB8R-FPV016). Supernatants of radiolabeled CV-1 cells infected with VVΔB8R-FPV016 contained a 34-kDa protein that specifically bound to matrix-immobilized chIFN-γ (Fig. 5A). This protein was not present in supernatants of CV-1 cells infected with the vaccinia virus strain VVΔB8R that carries no FPV genes (Fig. 5A). The 38-kDa IFN-γ-binding protein present in supernatants of FPV-infected cells (Fig. 2) was not observed in supernatants of VVΔB8R-FPV016-infected CV-1 cells. We do not know whether it was absent or whether its visualization was obscured by a background band of similar size (Fig. 5A).

To show that the recombinant FPV016 protein can neutralize the activity of chIFN-γ, we compared the activities of the supernatant of CV-1 cells infected with either VVΔB8R-FPV016 or VVΔB8R. Three different dilutions of supernatant (1:5, 1:10, and 1:20) were incubated with 10 units/ml chIFN-γ for 1 h before the mixtures were added to CEC-32 indicator cells. At 16 h post cytokine treatment, RNA was isolated and subjected to Northern blot analysis using a radioactive GBP cDNA probe. The supernatant of VVΔB8R-FPV016-infected cells clearly neutralized the activity of chIFN-γ (Fig. 5B). It blocked the GBP-inducing activity of chIFN-γ almost completely when used at a 1:5 dilution, and it was partially effective at 1:10 and 1:20. Control supernatant of VVΔB8R-infected cells did not block the GBP-inducing activity under these experimental conditions (Fig. 5B). At a 1:10 dilution, the supernatant of VVΔB8R-FPV016-infected cells had no detectable neutralizing effect on the antiviral activity of chIFN-α at any concentration that we tested (data not shown).

We next determined whether the FPV016 gene product might bind and neutralize IFN-γ from other species. The amino acid sequences of duck and chicken IFN-γ are 67% identical, and both proteins are active on quail cells (47). The supernatant of VVΔB8R-FPV016-infected cells neutralized the activity of duck IFN-γ with remarkably good efficacy, whereas the control supernatant of VVΔB8R-infected cells did not (Fig. 6A).

Interestingly, the supernatant of VVΔB8R-FPV016-infected cells also neutralized the activity of human IFN-γ (Fig. 6B), but it did not neutralize mouse (Fig. 6C) and rat (Fig. 6D) IFN-γ.

**DISCUSSION**

Sequence analysis of the complete FPV genome (40) yielded no evidence for the existence of a gene for a soluble IFN-γ-binding protein in this virus. Nevertheless, our experiments with the supernatants of FPV-infected chicken embryo cells clearly showed the presence of an activity that neutralized chIFN-γ (Fig. 1A). The simplest explanation for these discrepant results was that an FPV-encoded protein with no significant homology to previously described IFN-γ receptors exhibited IFN-γ-neutralizing activity. Our biochemical approach described in this paper demonstrated that this assumption was correct and that the FPV016 gene is encoding the critical viral factor.

The calculated molecular mass of the secreted form of the FPV016 gene product is ~24,000. However, our SDS gel analysis of viral proteins with high affinity for matrix-immobilized chIFN-γ showed that the factor in question presented itself as a pair of diffuse bands with apparent molecular masses of about 32–34 and 38–42 kDa (Fig. 2). This difference in molecular mass probably resulted from glycosylation of the mature protein. In fact, sequence analysis showed that the FPV016 protein has three potential N-glycosylation sites and one potential O-glycosylation site. Strong glycosylation of soluble IFN-γ-binding proteins of other poxviruses has been described (22, 23). It is of interest to note that our MALDI-TOF MS analysis of two gel spots with slightly different migration prop-
viral and cellular IFN-binding immunoglobulin domain. This was unexpected, because all known motifs in the FPV016 protein revealed the presence of an im-
tal strain did not. A computer search for characteristic protein whereas supernatants of control cells infected with the paren-
cines clearly showed that they both contained proteins encoded by the FPV016 gene and that one peptide, which presumably contains a glycosylated threonine residue, was missing in one of these proteins. Because the extent of glycosylation cannot be predicted, MALDI-TOF MS analysis most likely failed to detect the glycosylated peptide. These results suggested that O-glycosylation at threonine 93 does occur but that it is probably incomplete. Differences in glycosylation at this and other putative glycosylation sites of the FPV016 gene protein might further explain its appearance in multiple forms on two-dimen-
sional gels (Fig. 3).

The FPV016 gene is localized in the terminal region of the viral genome. This localization is characteristic for genes that code for immunomodulatory proteins in other poxviruses (2). By expressing the FPV016 gene with the help of a vaccinia virus that lacks its own soluble IFN-binding protein, we verified that the FPV016 gene product is indeed active. Supernatants of host cells infected with the FPV016-expressing vaccinia virus contained chIFN-γ binding and neutralizing activity, whereas supernatants of control cells infected with the parental strain did not. A computer search for characteristic protein motifs in the FPV016 protein revealed the presence of an immunoglobulin domain. This was unexpected, because all known viral and cellular IFN-γ receptors contain fibronectin type III domains rather than immunoglobulin domains (39). We thus identified a new type of viral IFN-γ-binding protein. This finding readily explains why our previous experiments failed to reveal the identity of the FPV016 gene product. Those attempts were all based on the assumption that IFN-γ-binding proteins of FPV and other poxviruses have fundamentally different primary structures. It is further possible that chickens possess an as yet unidentified gene that codes for a soluble IFN-γ-binding protein that is used to regulate the IFN-γ response and that FPV has pirated this gene.

FPV and other avian poxviruses are presently being evaluated as vaccine vectors for use in humans. These viral vectors are considered to be safe because they fail to replicate produc-
tively in mammalian cells. Our finding that FPV016 is capable of binding and neutralizing human IFN-γ (Fig. 6B) is relevant in this context. Because early IFN-γ synthesis is known to drive TH1-type immune responses, it is conceivable that FPV vaccine vectors that lack the 016 gene and, as a consequence, permit the accumulation of higher local concentrations of active IFN-γ might exhibit enhanced performance.

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