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Vaccinia, Cowpox, and Camelpox Viruses Encode Soluble Gamma Interferon Receptors with Novel Broad Species Specificity

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Soluble receptors for gamma interferon (IFN-γ) are secreted from cells infected by 17 orthopoxviruses, including vaccinia, cowpox, rabbitpox, buffalopox, elephantpox, and camelpox viruses, representing three species (vaccinia, cowpox, and camelpox viruses). The B8R open reading frame of vaccinia virus strain Western Reserve, which has sequence similarity to the extracellular binding domain of cellular IFN-γ receptors (IFN-γRs), is shown to encode an IFN-γ binding activity by expression in recombinant baculovirus. The soluble virus IFN-γRs bind IFN-γ and, by preventing its interaction with the cellular receptor, interfere with the antiviral effects induced by this cytokine. Interestingly, in contrast to cellular IFN-γRs, which are highly species specific, the vaccinia, cowpox, and camelpox virus IFN-γRs bind and inhibit the biological activity of human, bovine, and rat IFN-γ but not mouse IFN-γ. This unique broad species specificity of the IFN-γR would aid virus replication in different species and suggests that vaccinia, cowpox, and camelpox viruses may have evolved in several species, possibly including humans but excluding mice. Last, the conservation of an IFN-γR in orthopoxviruses emphasizes the importance of IFN-γ in defense against poxvirus infections.

Poxviruses are complex cytoplasmic DNA viruses that normally produce an acute infection in the host (14, 33). Members of the *Poxviridae* family are classified in genera, such as *Orthopoxvirus* (cowpox, vaccinia, variola, and camelpox viruses) and *Leporipoxvirus* (myxoma and Shope fibroma viruses). Variola virus, which caused smallpox, was a strictly human pathogen and produced a devastating disease with case fatality rates of up to 40% (15). Cowpox virus, so named because it was isolated from lesions on infected cattle, was introduced by Jenner in 1798 as a smallpox vaccine. However, the virus is not frequently found in cattle now, nor was it during the 18th and 19th centuries (5, 16). Sporadic infections in cows, humans, a wide range of zoo animals, and domestic cats are considered rare zoonoses, and the natural reservoir of cowpox virus may be wild rodents (5, 16). In this century, vaccinia virus, not cowpox virus, was the vaccine used to achieve the global eradication of smallpox by 1977 (15), but its origin and natural host are unknown (5, 15, 16). Following the extensive use of vaccinia virus for smallpox vaccination, the virus has infected domestic animals and buffalos, which in turn could transmit the virus to humans. Despite this, the virus is not considered a natural human pathogen. Previous suggestions that vaccinia virus might have derived from either cowpox or variola virus are now considered unlikely following analyses of their biological properties, restriction enzyme maps, and DNA sequences (1, 16, 17, 27). Camelpox virus has a narrow host range and causes a natural systemic infection in camels (16).

Poxviruses have acquired genes encoding proteins that modulate the host response to infection (7, 31, 33, 40). Some of these encode homologs of the extracellular binding domain of cytokine receptors that are secreted from infected cells and intercept the normal activities of particular cytokines. In the *Leporipoxvirus* genus, a soluble tumor necrosis factor receptor (TNFR) is secreted from cells infected by Shope fibroma and myxoma viruses and contributes to the virulence of myxoma virus, which produces myxomatosis in rabbits (39, 42). Myxoma virus also encodes a 37-kDa soluble gamma interferon receptor (IFN-γR) that binds and blocks the antiviral activity of rabbit IFN-γ (43). In the *Orthopoxvirus* genus, vaccinia and cowpox viruses express a soluble interleukin-1β receptor (IL-1βR) that modulates the host response to infection (3, 41), and cowpox virus expresses a soluble TNFR (20). Characterization of these virus cytokine receptors is increasing our understanding of poxvirus-host interactions.

The open reading frame (ORF) B8R protein products from vaccinia virus strains Western Reserve (WR) and Copenhagen show sequence similarity (21% amino acid identity) to the myxoma and Shope fibroma virus IFN-γRs and the extracellular domain of the mouse and human IFN-γRs (17, 19, 43). The B8R protein lacks the cytoplasmic and membrane anchor domains of the cellular IFN-γRs and thus is predicted to be secreted from vaccinia virus-infected cells and, as described for the myxoma virus IFN-γR (43), to bind and block the biological activities of this cytokine. IFN-γ is a pleiotropic cytokine that, besides inducing an antiviral state in cells, plays an important role in regulating immune and inflammatory processes (13, 37). The importance of IFN-γ in combating poxviral infections has been shown both in tissue culture and in animal models (21, 23, 24, 32). IFN-γ exerts its effects by binding with high affinity to a membrane-bound receptor in a species-specific manner (37). Thus, the analysis of the species specificity of the virus-encoded IFN-γR could give information on the natural host(s) in which the virus has evolved.

Here we report that soluble IFN-γRs are produced by 17 orthopoxviruses, including three distinct species (vaccinia, cowpox, and camelpox viruses). We show that an IFN-γ binding activity is encoded by the B8R ORF of vaccinia virus WR strain. The species specificity of the vaccinia, cowpox, and camelpox virus IFN-γRs is described and discussed in relation to the origin and natural host(s) of these viruses.

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MATERIALS AND METHODS

Cells and viruses. Human U937 cells were grown in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS). Human TK⁻143B and HeLa

cells, mouse L929 cells, and bovine MDBK cells were grown in minimal essential medium (Gibco) supplemented with 10% FBS.

The WR strain of vaccinia virus was obtained from B. Moss (National Institutes of Health, Bethesda, Md.). The Tashkent, IHD-J, and IHD-W strains of vaccinia virus and the Brighton Red strain of cowpox virus were obtained from M. Mackett (Paterson Institute for Cancer Research, Manchester, United Kingdom [UK]) and J. D. Williamson (St. Mary's Hospital Medical School, London, UK). The New York City Board of Health vaccine strain (Wyeth) was obtained from Wyeth Laboratories, and the Lister strain was obtained from Vestric Limited. Rabbitpox virus was provided by R. W. Moyer (University of Florida, Gainsville). The Temple of Heaven strain (Tian-Tan) and the Copenhagen strain were obtained from J. Zhou (Princess Alexandra Hospital, Brisbane, Australia) and R. Drillien (University Louis Pasteur, Strasbourg, France), respectively. The vaccinia virus strains King Institute, USSR, Dairen, and Patwadangar and buffalopox, elephantpox, and camelpox viruses were obtained from D. Baxby (University of Liverpool, Liverpool, UK) and K. R. Dumbell (University of Cape Town, Cape Town, South Africa). Cocal virus (22) was obtained from W. James (Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom).

Spodoptera frugiperda (Sf) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were cultured in TC100 medium (Gibco) containing 10% FBS. The baculovirus recombinant AcB15R, expressing the vaccinia virus IL-1βR, has been described before (3).

Reagents. Radioiodinated human recombinant IFN-γ (^{125}I-hIFN-γ; 50 to 100 μCi/μg) was obtained from Du Pont-New England Nuclear. Purified recombinant human and mouse IFN-γ (10^7 U/mg) were purchased from Genzyme. Purified recombinant rat and bovine IFN-γ (4×10^6 U/mg) were obtained from Gibco and R. A. Collins (Institute of Animal Health, Compton, United Kingdom), respectively.

Construction of recombinant baculovirus. Plasmid pSTH1 (18), containing an *Eco*RI-*Sal*I fragment (ORF B5R to B13R) of vaccinia virus WR, was digested with *Dra*I and *Hin*cII, and a 922-bp fragment which contains ORF B8R and 48 and 45 nucleotides of the 5′- and 3′-flanking regions, respectively, was isolated. This fragment was cloned into *Sal*I-digested and Klenow enzyme-treated pUC4K (45), and the resulting plasmid was termed pAA22. The B8R gene was excised from pAA22 with *Bam*HI and inserted into *Bam*HI-cut pAcRP25 (30), forming pAA23. Sf cells were cotransfected with purified linear AcNPV DNA (BacPAK6, Clontech Laboratories) and pAA23 following the instructions provided by the manufacturer, and a recombinant virus was isolated. This was termed AcAA5, but is referred to hereafter as AcB8R.

Metabolic labeling of proteins and electrophoretic analysis. Sf cells were infected with the baculovirus recombinants at a multiplicity of infection of 10 PFU per cell. After 24 h of infection, cultures were pulse labeled with 200 μCi of ^{35}STrans-label (ICN biomedicals; 1,200 Ci/mmol) per ml in methionine- and cysteine-free TC100 medium in the absence of serum. Cells or medium was dissociated in sample buffer containing 0.4 M Tris-HCl (pH 6.3), 2.3% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% 2-mercaptoethanol and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 12% acrylamide gels. Proteins were visualized by fluorography with Amplify (Amersham) or Coomassie blue staining.

Preparation of medium for binding and biological assays of IFN-γ. Cultures of TK$^-$143B cells or Sf cells were infected with a multiplicity of infection of 10 PFU per cell in serum-free medium. Supernatants from orthopoxvirus- or baculovirus-infected cells were harvested at 1 or 3 days postinfection, respectively, and centrifuged at 3,000 rpm for 10 min at 4°C; the pellet was discarded, and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4) was added to a final concentration of 20 mM. The medium from 1×10^3 to 3.5×10^3 cells per ml was stored at −70°C until used in binding assays. Supernatants used in biological assays were centrifuged at 16,500 rpm in an SW41Ti rotor for 60 min to remove virus particles and subsequently dialyzed and concentrated against phosphate-buffered saline at 4°C in a Micro-ProDiCon (Bio-Molecular Dynamics) with PA-10 ProDiMen dialysis membranes (molecular weight cutoff, 10,000) to a final concentration equivalent to 6×10^7 cells per ml.

Binding assays. The binding medium used in the different assays was RPMI 1640 containing 20 mM HEPES (pH 7.4) and 1% FBS. In the competition assays with U937 cells, supernatants were preincubated with 200 pM ^{125}I-hIFN-γ in 125 μl for 1 h at 4°C. Subsequently, 2.5×10^6 U937 cells were added in 25 μl and incubated for 2 h at 4°C. Bound ^{125}I-hIFN-γ was determined by phthalate oil centrifugation as described before (12) and was 5×10^3 to 9×10^3 cpm in the absence of competitor. Cross-linking experiments with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) to ^{125}I-hIFN-γ were performed as described before (43). Briefly, supernatants were incubated in 20 μl with 2 nM radioactive ^{125}I-hIFN-γ at room temperature for 2 h. The cross-linking agent was added to 40 mM final concentration, incubated at room temperature for 30 min, and quenched by addition of 0.1 M Tris (pH 7.5). Samples were centrifuged at 13,000 × *g* for 15 min and analyzed by SDS-PAGE in 12% acrylamide gels.

Activity assay for IFN-γ. The biological activity of IFN-γ was measured by the inhibition of cocal virus plaque formation. Human, bovine, and mouse IFN-γ were assayed in cultures of human HeLa cells, bovine MDBK cells, and mouse L929 cells, respectively. The antiviral activity of rat IFN-γ was assayed in monolayers of mouse L929 cells, since the mouse IFN-γR binds rat IFN-γ (44). Cell monolayers were pretreated in 0.5 ml of medium containing 10% FBS, IFN-γ, and virus-free supernatants. After 16 to 24 h at 37°C, monolayers were rinsed and infected with approximately 100 PFU of cocal virus, and plaques were counted after 2 days. The concentrated supernatants used as a source of secreted proteins did not produce plaques (data not shown).

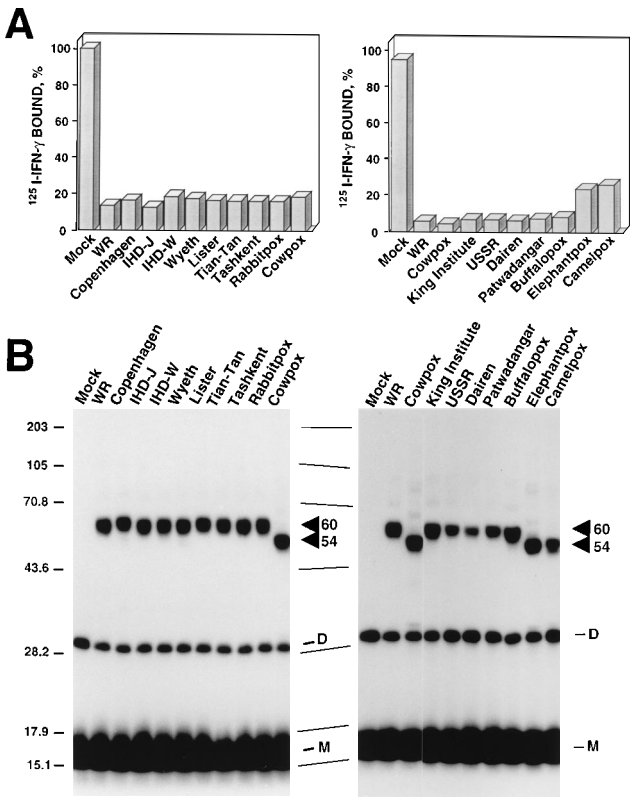

FIG. 1. IFN-γ binding activity secreted from cultures infected with different orthopoxviruses. (A) Competition of IFN-γ binding to U937 cells. Medium from cultures infected with different viruses (equivalent to 1×10^5 to 1.7×10^5 cells) was incubated with ^{125}I-hIFN-γ for 1 h at 4°C. U937 cells were added and incubated for 2 h at 4°C, and the radioactivity bound to the cells was determined by phthalate oil centrifugation. Data are expressed as the percentage of counts binding in the presence of competitor compared with that without competitor. (B) Cross-linking of ^{125}I-hIFN-γ to medium from cultures infected with different viruses. The amount of supernatant added was equivalent to 1.5×10^4 to 3.5×10^4 cells. An autoradiograph of the SDS-PAGE analysis (with molecular masses in kilodaltons) is shown. The positions of the IFN-γ monomer (M), IFN-γ dimer (D), and ligand-receptor complexes (◀) are indicated.

RESULTS

Binding activity for IFN-γ in the supernatants of cultures infected with orthopoxviruses. To analyze the expression of soluble IFN-γRs by different orthopoxviruses, supernatants from infected cultures were harvested at 24 h postinfection and tested for their capacity to inhibit the binding of ^{125}I-hIFN-γ to human U937 cells. Figure 1A shows that medium from cultures infected with 12 strains of vaccinia, rabbitpox, or buffalopox viruses (considered vaccinia virus strains), cowpox virus (Brighton Red strain), elephantpox virus (considered a cowpox virus strain), or camelpox virus prevented the binding of the radiolabeled cytokine to the cells, suggesting that an IFN-γ binding activity is secreted from these virus-infected cultures. The inhibitory activity was not due to degradation of the labeled cytokine, since the ^{125}I-hIFN-γ remained intact in the medium after incubation with the supernatants, as determined by SDS-PAGE (data not shown).

To provide direct evidence of the presence of an IFN-γ

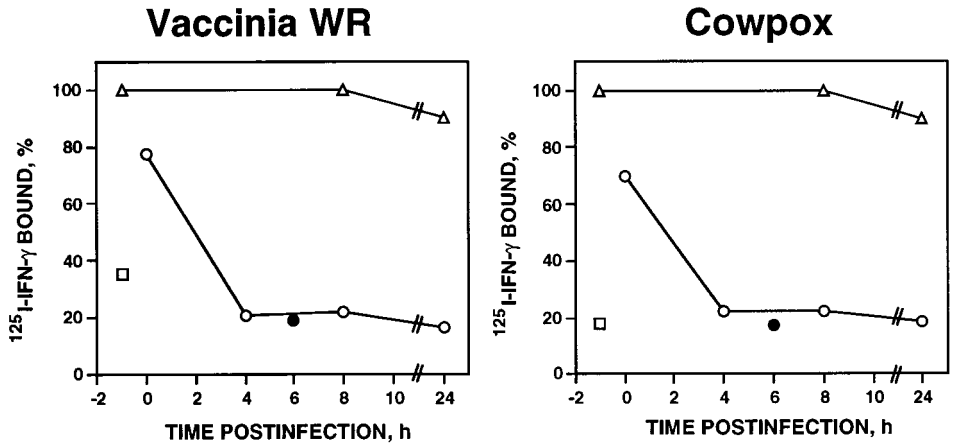

FIG. 2. Kinetics of synthesis of the IFN-γ binding activity. Monolayers of TK$^-$143B cells were mock-infected (△) or infected with 10 PFU of vaccinia virus WR or cowpox virus, in the absence (○) or in the presence (●) of 40 μg of cytosine arabinoside per ml. After 1 h of adsorption, cells were rinsed twice with medium and incubated for different times. At the indicated time postinfection, supernatants were harvested, and an aliquot (equivalent to 1.7×10^5 cells) was tested for its capacity to block the binding of IFN-γ to U937 cells. Medium samples were incubated with ^{125}I-hIFN-γ for 1 h at 4°C before addition of U937 cells and further incubation for 2 h at 4°C. The radioactivity bound to the cells was determined by phthalate oil centrifugation. The dose of virus inoculum used to infect 1.7×10^5 cells was also tested in the competition assay (□). Data are expressed as the percentage of counts binding in the presence of competitor compared with that without competitor.

binding protein in the supernatants, ^{125}I-hIFN-γ was covalently cross-linked to the soluble receptor as described before (43). Figure 1B shows that, in contrast to uninfected supernatants, in which only monomers and homodimers of IFN-γ were detected, a labeled higher-molecular-mass complex was observed in supernatants from all virus-infected cultures tested. The size of the ligand-receptor complex in the supernatants of all vaccinia virus strains was 60 kDa, which, after subtraction of the size of the IFN-γ monomer (17 kDa), suggests a receptor size of 43 kDa. Although IFN-γ is a noncovalent homodimer, only cross-linked heterodimers of IFN-γ and virus receptor were detected, as described previously (43). Interestingly, the predicted size of the vaccinia virus IFN-γR (43 kDa) was higher than that reported for the myxoma virus IFN-γR (37 kDa [43]). The observed size difference of the myxoma and vaccinia virus proteins might reflect different degrees of glycosylation, since the vaccinia virus WR and Copenhagen polypeptides are predicted to be 31 kDa and possess one more potential N-glycosylation site than the predicted myxoma virus protein of 30 kDa (17, 19, 43). The cowpox and camelpox virus receptor-ligand complexes had a size of 54 kDa, suggesting a receptor size of 37 kDa. Rabbitpox and buffalopox viruses had a receptor size typical of vaccinia virus, consistent with the proposal that these viruses are vaccinia virus strains (16). Similarly, elephantpox virus has an IFN-γR of the same size as cowpox virus, and elephantpox virus is thought to be a cowpox virus strain (16).

Binding of ^{125}I-hIFN-γ to cells infected with vaccinia virus WR or Lister or cowpox virus did not increase compared with that in uninfected cultures (data not shown), indicating that the virus IFN-γR is not anchored in the plasma membrane.

Kinetics of synthesis of the vaccinia and cowpox virus IFN-γRs. The kinetics of production of soluble IFN-γR from cells infected with vaccinia virus strain WR or Lister or cowpox virus was examined by testing the ability of supernatants harvested at different times after infection to inhibit the binding of ^{125}I-hIFN-γ to U937 cells. Figure 2 shows that the soluble IFN-γ binding activity expressed by vaccinia virus WR and cowpox virus was abundant 4 h after infection and competitively inhibited binding of most of the IFN-γ to U937 cells. The presence of the activity in cultures infected in the presence of cytosine arabinoside, an inhibitor of DNA synthesis, demonstrated that the protein is translated at early times of infection, before viral DNA replication takes place. The experiment shown in Fig. 2 did not determine if the receptor continued to be synthesized at late times of infection, since maximum competition was already observed at early times of infection. However, repetition of the competition assay with lower doses of supernatant (equivalent to up to 7×10^3 cells per assay) showed that the receptor did not accumulate between 6 and 24 h postinfection (data not shown), which suggested that the protein is only produced at early times of infection. Similar results were obtained with the vaccinia virus Lister strain (data not shown).

B8R ORF of vaccinia virus WR strain encodes a soluble IFN-γ binding activity. The product of the B8R ORF of vaccinia virus WR shows sequence similarity with the myxoma virus IFN-γR and the extracellular domain of the cellular IFN-γRs. To further characterize the products of the B8R ORF, a recombinant baculovirus termed AcB8R was constructed, in which the B8R gene was transcribed under the control of the polyhedrin promoter. Pulse-label experiments showed that the B8R protein was secreted into the medium of AcB8R-infected Sf cells as a polypeptide of 32 to 35 kDa (Fig. 3A), which was not expressed by AcNPV or a recombinant baculovirus expressing the vaccinia virus IL-1βR (AcB15R [3]). Incomplete glycosylation of the polypeptide in insect cells (25) might contribute to a size smaller than that estimated for the receptor secreted from vaccinia virus-infected cells (43 kDa; Fig. 1B). The intracellular 31-kDa form correlated with the predicted size of the nascent B8R polypeptide and thus may represent the unglycosylated protein, and the intracellular 34- and 38-kDa species may represent different degrees of glycosylation. Similarly, expression of the vaccinia virus IL-1βR (B15R) in the baculovirus system produced several intracellular forms and a secreted form smaller than the 50- to 60-kDa protein expressed from mammalian cells (Fig. 3A) (3). The B8R protein was very abundant in the supernatants of AcB8R-infected insect cultures and was clearly detected by Coomassie staining (Fig. 3B).

The capacity of the baculovirus-expressed B8R protein to bind ^{125}I-hIFN-γ was tested in a cross-linking assay (Fig. 4A). A 52-kDa ligand-receptor complex was observed in superna-

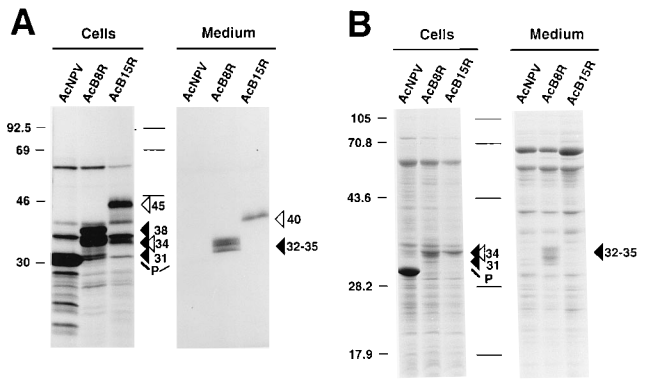

FIG. 3. Expression of B8R protein in baculovirus-infected insect cells. (A) Metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine. Sf cells infected with AcNPV, AcB8R, or AcB15R were pulse-labeled with ^{35}STrans-label for 2 h after 24 h of infection. Proteins present in cells and medium were analyzed by SDS-PAGE and visualized by fluorography. Twice as much material was loaded onto gels from supernatants as from cells, and the fluorograph was exposed twice as long. The positions of polyhedrin (P) and the B8R (◀) and B15R (◁) gene products are indicated. Molecular masses are shown in kilodaltons. (B) Coomassie staining. Sf cells were infected with AcNPV, AcB8R, or AcB15R, and cells and medium were harvested 3 days after infection. Samples, 5×10^5 cells or medium equivalent to 9×10^5 cells, were analyzed by SDS-PAGE and stained with Coomassie blue. The positions of polyhedrin (P) and the B8R (◀) and B15R (◁) gene products are indicated. Molecular masses are shown in kilodaltons.

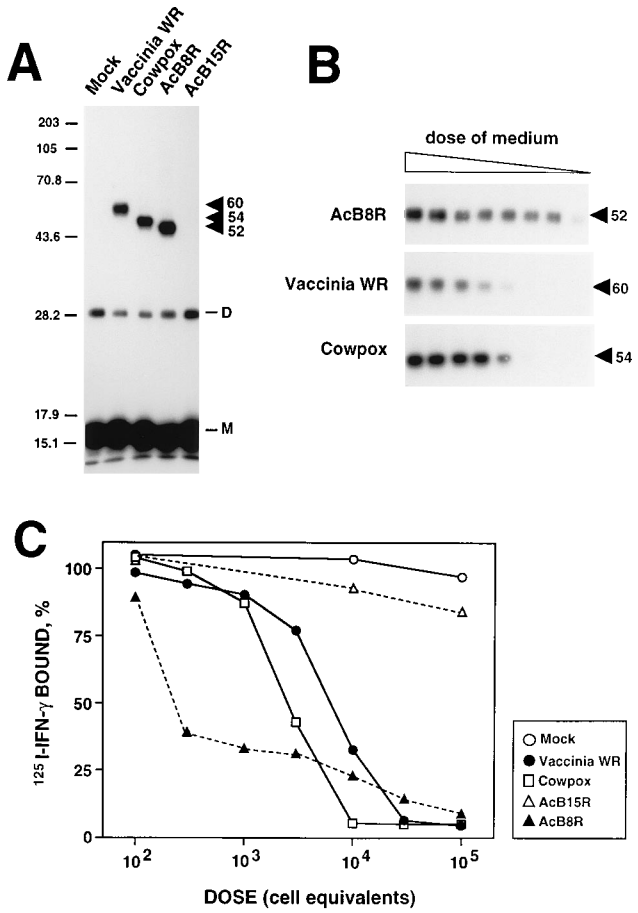

FIG. 4. IFN-γ binding activity of the B8R protein expressed in baculovirus-infected insect cells. (A) Cross-linking of ^{125}I-hIFN-γ to medium from mock-infected cultures or cultures infected with the indicated viruses. The amount of supernatant added was equivalent to 3.5×10^4 cells. An autoradiograph of the SDS-PAGE analysis (with molecular masses in kilodaltons) is shown. The positions of the IFN-γ monomer (M), IFN-γ dimer (D), and ligand-receptor complexes (◀) are indicated. (B) Cross-linking of ^{125}I-hIFN-γ to different amounts of medium (equivalent to 5×10^5, 1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3, 5×10^2, and 1×10^2 cells) from cultures infected with the indicated viruses. An autoradiograph of the SDS-PAGE is shown. The positions of the ligand-receptor complexes (◀) and molecular masses in kilodaltons are indicated. (C) Competition of IFN-γ binding to U937 cells. Different amounts of medium from cultures of Sf cells infected with AcB15R (△) or AcB8R (▲) or TK$^-$143B cells mock-infected (○) or infected with vaccinia virus WR (●) or cowpox virus (□) were incubated with ^{125}I-hIFN-γ for 1 h at 4°C. U937 cells were added and incubated for 2 h at 4°C, and the radioactivity bound to the cells was determined by phthalate oil centrifugation. Data are expressed as the percentage of counts binding in the presence of competitor compared with that without competitor.

tants from cultures infected with AcB8R but not AcB15R, and this size was consistent with the 32- to 35-kDa receptor being cross-linked with the 17-kDa IFN-γ. The size of the complex was smaller than those of vaccinia and cowpox viruses (Fig. 4A). The ^{125}I-hIFN-γ-receptor complex was detected after addition of supernatants from 5×10^3, 1×10^3, or 1×10^2 cells from vaccinia virus WR-, cowpox virus-, and AcB8R-infected cultures, respectively, illustrating the high expression level of the baculovirus system (Fig. 4B). In similar experiments, the relative amount of IFN-γ binding activity produced by camelpox virus was the same as that produced by vaccinia virus WR (data not shown).

The binding activity of the receptor expressed by recombinant baculovirus was also investigated by competitively inhibiting the binding of IFN-γ to U937 cells. Figure 4C shows that ^{125}I-hIFN-γ binding to U937 cells was inhibited in a dose-dependent manner by supernatants of insect cells containing the B8R protein but not the vaccinia virus IL-1βR. Higher doses of supernatants from cowpox virus- and vaccinia virus WR-infected cultures were required to inhibit the binding, consonant with the quantification observed in cross-linking experiments (Fig. 4B). However, the baculovirus-produced B8R protein was less efficient than the mammalian-expressed protein in achieving maximum competition.

Species specificity of the vaccinia virus and cowpox virus IFN-γ binding activity. With a few exceptions, IFN-γ interacts exclusively with receptors from the same species (37). Human IFN-γ does not bind to mouse, rat, or bovine cells, and mouse IFN-γ does not bind to human cells (37). Bovine IFN-γ does not have any biological effects on human, rat, or mouse cells, only bovine cells (9). Rat IFN-γ has no activity on human cells, but its high similarity to mouse IFN-γ (87% amino acid identity) probably explains its activity on mouse cells. However, conversely, mouse IFN-γ is not active in rat cells (11, 44).

The species specificity of the virus receptor was investigated by cross-linking with ^{125}I-hIFN-γ in the presence of increasing concentrations of unlabeled IFN-γ from different species. Figure 5 shows that similar concentrations of unlabeled human, bovine, and rat IFN-γ efficiently inhibited the binding of ^{125}I-hIFN-γ to the soluble receptor expressed from vaccinia virus WR-, cowpox virus-, camelpox virus-, or AcB8R-infected cells, suggesting similar affinities for each receptor-cytokine interaction. In contrast, very high concentrations of mouse IFN-γ did not inhibit the binding of ^{125}I-hIFN-γ to the vaccinia virus receptor, expressed from either vaccinia virus WR or recombinant baculovirus, or to the cowpox virus receptor. With the camelpox virus receptor, the relatively weak inhibition of ^{125}I-hIFN-γ binding by mouse IFN-γ suggested a higher, although probably poor, affinity for the mouse cytokine.

Similar competition assays, with a 100-fold excess of unlabeled IFN-γ, showed that the IFN-γRs from all the orthopoxviruses included in Fig. 1 had the same species specificity as

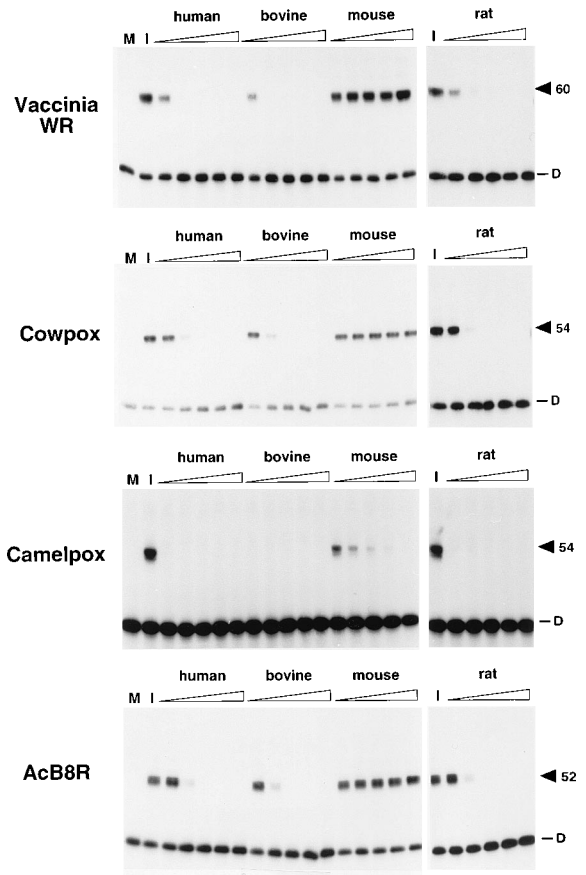

FIG. 5. Species specificity of the IFN-γR encoded by vaccinia, cowpox, and camelpox viruses: cross-linking of 2 nM ^{125}I-hIFN-γ to medium from uninfected (M) or infected (I) cultures in the absence or in the presence of increasing concentrations of unlabeled IFN-γ from different species. The amount of medium used in the assay was equivalent to 9×10^4 cells from TK$^-$143B cell cultures infected with vaccinia virus WR and cowpox virus, 1.5×10^3 cells from TK$^-$143B cells infected with camelpox virus, and 3×10^3 cells from insect cell cultures infected with AcB8R. The concentrations of unlabeled human, bovine, mouse, and rat IFN-γ added were 10-, 20-, 50-, 100-, and 300-fold excess. An autoradiograph of the SDS-PAGE analysis (with molecular masses in kilodaltons) is shown. The positions of the IFN-γ dimer (D) and ligand-receptor complexes (◀) are indicated.

vaccinia virus WR, except that the binding of ^{125}I-hIFN-γ to the elephantpox virus receptor was partially inhibited by mouse IFN-γ (data not shown). Further experiments with elephantpox virus and various doses of mouse IFN-γ gave results similar to those for camelpox virus (data not shown).

The capacity of the virus receptors to inhibit the antiviral activity induced by IFN-γ from different species was tested in a plaque assay with cocal virus, a rhabdovirus, in cells of the appropriate species. In this assay, addition of IFN-γ inhibits the formation of plaques by cocal virus, and the presence of biologically active virus IFN-γR can be detected by plaque formation. The IFN-γR from cultures infected with vaccinia virus WR, cowpox virus, AcB8R, camelpox virus, and elephantpox virus was a potent inhibitor of human, bovine, and rat but not mouse IFN-γ (Fig. 6). The mouse cytokine was not even inhibited by a 200-fold-greater dose of AcB8R-infected supernatants than was needed to inhibit human IFN-γ (data not shown). Higher concentrations of bovine IFN-γ were required to inhibit cocal virus replication, and, consistently, higher doses of receptor were needed to block the activity. The high abundance of the soluble IFN-γR was illustrated by the inhibition of 10 U of human IFN-γ per ml by 0.3, 3, or 15 μl of medium from AcB8R-, cowpox virus-, or vaccinia virus WR-infected cultures, respectively. These results were consistent with those of the binding and competition assays shown in Fig. 4B and C except that, previously, complete inhibition of ^{125}I-hIFN-γ binding by the baculovirus-expressed protein was only seen at high doses. Although cross-linking competition binding experiments suggested a higher affinity of the camelpox and elephantpox virus receptors for mouse IFN-γ, the failure of high doses of these IFN-γRs to inhibit the biological activity of the mouse cytokine suggested that the affinity is too low to block mouse IFN-γ under physiological conditions (Fig. 6).

DISCUSSION

IFNs (α, β, and γ) induce an antiviral state by upregulating the synthesis of several host proteins. In addition, IFN-γ is an important regulator of cell-mediated mechanisms of host defense (13). In this paper, we show that soluble IFN-γRs are secreted from cells infected with three distinct species of orthopoxvirus (vaccinia, cowpox, and camelpox viruses) and that the B8R ORF of vaccinia virus WR encodes an IFN-γ binding activity. Although not formally proven, B8R is likely to be the only IFN-γR encoded by vaccinia virus because (i) the complete sequence of vaccinia virus contains only a single ORF (B8R) encoding a protein with amino acid similarity to the extracellular domain of IFN-γRs (17); (ii) the binding characteristics of the B8R protein expressed from recombinant baculovirus for IFN-γ from several species are indistinguishable from those present in the supernatant of vaccinia virus-infected cells; and (iii) cross-linking experiments with ^{125}I-labeled IFN-γ detected only a single band in the supernatants of cells infected with 14 strains of vaccinia virus.

The B8R protein is the third vaccinia virus protein shown to counteract the antiviral effects of IFNs. ORFs K3L and E3L encode intracellular proteins that block the IFN-induced inhibition of protein synthesis (6, 8, 10). In contrast, the B8R protein is secreted, binds soluble IFN-γ, and inhibits IFN-γ activity by preventing its interaction with cellular receptors. This strategy to blockade IFN-γ allows poxviruses to inhibit both the antiviral and immune functions of IFN-γ simultaneously. Similarly, soluble IFN-γRs have been found in human urine and may function as regulators of IFN-γ activity in vivo (36).

All three of the anti-IFN proteins encoded by vaccinia virus, the cowpox virus TNFR (20), and the myxoma virus IFN-γR and TNFR (42, 43) are expressed early during infection, while the vaccinia virus soluble IL-1βR is expressed late (3). This illustrates the importance to poxviruses of counteracting those cytokines that have a direct effect on the infected cell immediately after infection. Similarly, adenovirus encodes several proteins that block the antiviral effects of TNF, and each is expressed from early transcription units (46).

The IFN-γR is highly conserved among 17 orthopoxviruses, including 12 strains of vaccinia virus (nine of which were smallpox vaccines), two strains of cowpox virus, and camelpox virus. In addition, an IFN-γR gene has been found in two strains of variola virus (29, 38) and swinepox virus (*Suipoxvirus* genus [28]), and an active IFN-γR is expressed by myxoma virus (*Leporipoxvirus* genus [43]). The expression of an IFN-γR in all orthopoxviruses tested contrasts with the distribution of TN FRs and IL-1βRs. The IL-1βR is absent in 3 of 14 strains of vaccinia virus tested (2, 3), and the corresponding gene is inactive in two variola virus strains (4, 29, 38). Two TNFR genes exist in vaccinia virus strains Copenhagen and WR, but

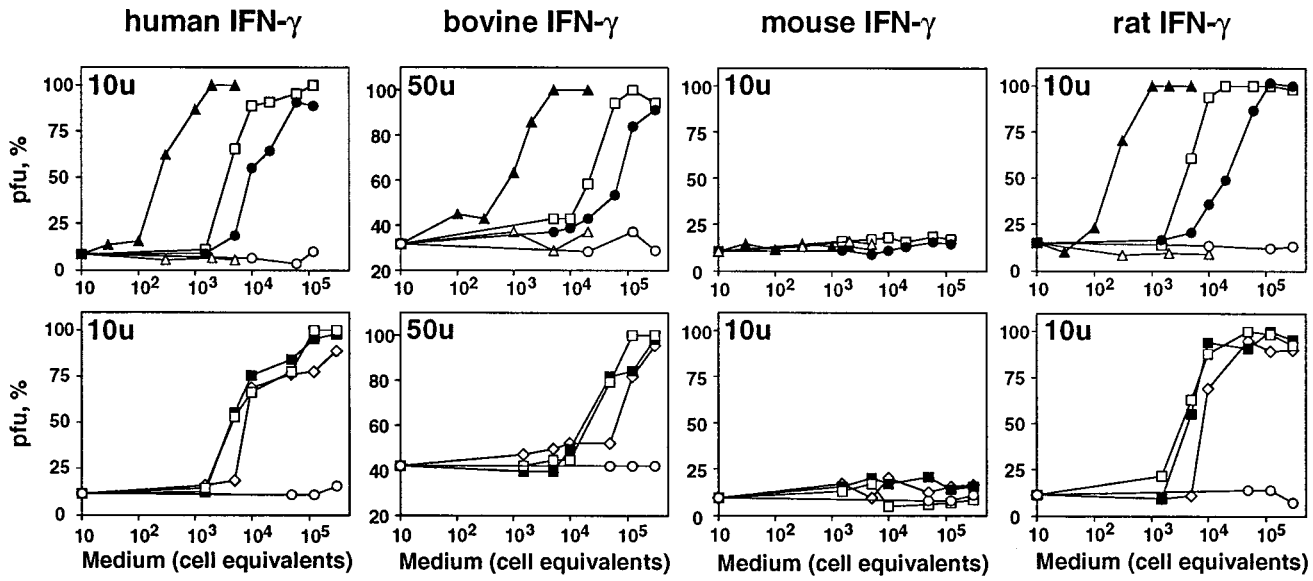

FIG. 6. Inhibition of the antiviral activity of IFN-γ by the virus IFN-γR. The indicated amounts (units per milliliter) of IFN-γ were tested in an antiviral activity assay with cocal virus in the absence or in the presence of various amounts of supernatants from TK$^-$143B cells mock-infected (○) or infected with vaccinia virus WR (●), cowpox virus (□), camelpox virus (◇), or elephantpox virus (■) or Sf cells infected with AcB15R (△) or AcB8R (▲). Human, bovine, and mouse IFN-γ were assayed in cultures of human HeLa cells, bovine MDBK cells, and mouse L929 cells, respectively. Rat IFN-γ was assayed in monolayers of mouse L929 cells (44). The percent PFU refers to the number of cocal virus plaques obtained in the presence of IFN-γ and increasing amounts of test medium compared with that without IFN-γ.

both are fragmented (17, 19, 42), and TNF binding activity is absent in 12 of 14 strains of vaccinia virus tested (2). One of the two TNFR homolog genes is deleted in variola virus, whereas the other is predicted to encode an active TNFR (1, 4, 29, 38), and this gene is active in cowpox virus (20).

The expression of a soluble IFN-γR by all orthopoxviruses tested suggests a major role in virus pathogenesis. Like the vaccinia virus IL-1βR and the myxoma virus TNFR (3, 41, 42), the IFN-γR is predicted to be nonessential for virus replication in tissue culture, and consequently, mutations leading to its inactivation might have accumulated during in vitro passage and without the selective pressure imposed by an animal host. However, only laboratory strains of vaccinia virus, such as WR, have been passed extensively in vitro, while smallpox vaccine strains were grown on the skin of animals (15, 16). The latter growth conditions may have provided selective pressure for retention of an IFN-γR.

The vaccinia, cowpox, and camelpox virus IFN-γRs have novel broad species specificity and inhibit the biological activity of human, bovine, and rat but not mouse IFN-γ. It is quite possible that the virus receptors bind IFN-γ from other species not included here. In a parallel study, the vaccinia virus IFN-γR has been found to bind human and rabbit IFN-γ and, very poorly, mouse IFN-γ (34). This property is consistent with the capacity of both vaccinia and cowpox viruses to infect a broad range of species. In contrast, the IFN-γR encoded by myxoma virus, a rabbit pathogen, binds rabbit but not human or mouse IFN-γ (35). However, variola virus, a strictly human pathogen, encodes a B8R homolog with 91% amino acid identity to the vaccinia virus ORF B8R product, much higher than the 19% amino acid identity of vaccinia virus B8R with the myxoma virus homolog, suggesting a broad species specificity similar to that of vaccinia virus. Similarly, camelpox virus infections occur only in camels, yet the studies presented here indicate a broad IFN-γ species specificity of the camelpox virus receptor. Although variola and camelpox viruses show a narrow host range in natural infections, limited virus replication has been reported in experimental animals (16), suggesting that other species were possible hosts for these viruses during evolution. Perhaps an orthopoxvirus ancestor had an IFN-γR with broad species specificity, a property that has been conserved in many members of the genus, although some of these have later evolved a more restricted host range.

The novel broad species specificity of the vaccinia and cowpox virus IFN-γRs has implications for the origin and natural host(s) of these viruses. The cowpox virus IFN-γR binds bovine IFN-γ, as expected, but not mouse IFN-γ, suggesting that the mouse is not a natural host. Conversely, the binding of rat IFN-γ supports the hypothesis that other rodents, such as susliks and gerbils, from which cowpox virus has been isolated (26), may constitute a natural reservoir. The ability of both the cowpox and vaccinia virus IFN-γRs to bind human IFN-γ suggests that, although these viruses are not considered human pathogens, they may have naturally infected humans in the past. Additionally, the similar properties of these IFN-γRs suggest that cowpox and vaccinia viruses may have evolved in the same host(s) and are consistent with the finding that all the smallpox vaccine strains used during the 20th century, isolated independently and originally thought to be cowpox virus, are in fact vaccinia virus. For example, horsepox virus, from which smallpox vaccines were established on several occasions, was eliminated from its natural host by the end of the 19th century and may well have been vaccinia virus, as proposed by Baxby (5).

In conclusion, vaccinia, cowpox, and camelpox viruses encode soluble IFN-γRs that counteract the activity of the cytokine and possess a broad species specificity. This novel property of the IFN-γR probably helped these orthopoxviruses to replicate in several species. This would have been an evolutionary advantage to these viruses, which are unable to perpetuate themselves in the population by establishing latent infections. The species specificity of the virus IFN-γR is also informative and indicates possible natural hosts of these viruses.

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