

Vaccinia Virus Encodes a Soluble Type I Interferon Receptor of Novel Structure and Broad Species Specificity

Julian A. Symons, Antonio Alcamí, and Geoffrey L. Smith

Sir William Dunn School of Pathology
University of Oxford
South Parks Road
Oxford OX1 3RE
England

Summary

Vaccinia virus (VV) and other orthopoxviruses express a soluble type I interferon (IFN) receptor that for VV strain Western Reserve is encoded by gene B18R. The 60–65 kDa glycoprotein is related to the interleukin-1 receptors and is a member of the immunoglobulin superfamily, unlike other type I IFN receptors, which belong to the class II cytokine receptor family. The receptor has high affinity (K_d , 174 pM) for human IFN α and, unlike other type I IFN receptors, has broad species specificity, binding to human, rabbit, bovine, rat, and mouse type I IFNs. This may have aided VV replication in multiple host species during evolution. A VV B18R deletion mutant is attenuated in a murine intranasal model. This type I IFN receptor represents the fourth VV protein that interferes with IFN and the fourth soluble cytokine receptor expressed by poxviruses.

Introduction

Interferons (IFNs) are a heterogeneous family of cytokines that were initially defined by their ability to induce resistance to viral infection (Isaacs and Lindermann, 1957). The various IFN α subtypes and IFN β (type I IFNs) share sequence similarity and bind common, species-specific receptors on host cells (for reviews, see Peska and Langer, 1987; Callard and Gearing, 1994). IFN γ (type II IFN) binds to a different receptor (Aguet et al., 1988). In addition to their antiviral effects, IFNs exhibit other biological properties, including regulation of cellular differentiation, proliferation, and immunomodulation.

Two human type I IFN receptors have been characterized. By use of a gene transfer approach, a 95–110 kDa type I membrane glycoprotein was identified that mediates responses to IFN α 8 but not to other human IFN α subtypes or to IFN β (Uze et al., 1990). More recently, a second type I IFN receptor was cloned after the identification, purification, and sequence analysis of a 40 kDa soluble form of the receptor. The membrane-associated form is a 51 kDa glycoprotein that is disulphide-bonded into a 102 kDa homodimer that binds to most IFN α subtypes and to IFN β (Novick et al., 1994). A mouse IFN α receptor related to the human 95–110 kDa receptor has also been cloned (Uze et al., 1992) that mediates the activities of all the mouse type I IFN subtypes. The type I IFN receptors possess two or four fibronectin type III domains and belong to the class II cytokine receptor family (CRF2) that includes

the receptors for IFN γ and interleukin (IL)-10 and the CRF2-4 gene product. These exhibit 20%–30% amino acid identity and share common motifs, including two cysteine pairs and conserved proline, tryptophan, and tyrosine residues (Callard and Gearing, 1994).

Targeted deletion of the mouse type I IFN receptor has revealed that the type I IFN system is essential for host defense against some viruses. Mutant mice became 10⁶-fold to 10⁷-fold more sensitive to vesicular stomatitis virus and Semliki Forest virus than normal mice (Muller et al., 1994). In contrast, inactivation of the type II IFN system had no effect on infection with these viruses. However, functional type I and type II IFN systems are required for defense against lymphocytic choriomeningitis virus and vaccinia virus (VV) (Muller et al., 1994).

VV is the prototypic member of the poxvirus family of cytoplasmic DNA viruses (Moss, 1990) and is the live vaccine used to eradicate smallpox (Fenner et al., 1988). The 191 kbp viral genome has approximately 200 genes (Goebel et al., 1990; Johnson et al., 1993) and is organized with essential genes, such as those encoding enzymes required for replication and transcription, clustered toward the genome center, whereas nonessential genes influencing host range and virulence map toward the termini. Several of these latter genes encode proteins involved in the evasion of the host immune response to infection (Smith, 1993).

IFN is important for protection against poxvirus infections. Mice with a targeted disruption of the type I or type II IFN system are more susceptible to infection by VV, despite a normal cytotoxic T cell response (Dalton et al., 1993; Huang et al., 1993; Muller et al., 1994). Treatment of mousepox virus-infected mice with neutralizing antibodies to either type I or type II IFN resulted in defective clearance of virus (Karupiah et al., 1993a), whereas treatment of mice with IFN prior to infection with VV abrogates the infection (Rodriguez et al., 1991). Furthermore, athymic nude mice infected with a recombinant VV expressing IFN γ recovered from infection, whereas mice infected with a wild-type VV died (Kohonen-Corish et al., 1990). Lastly, the inhibition of nitric oxide synthase in mice, which mediates the antiviral activity of IFN γ , converts a resolving infection into fulminant mousepox (Karupiah et al., 1993b).

Poxviruses have several strategies to counteract the action of IFN. The VV E3L protein competitively binds double-stranded RNA (dsRNA) and prevents the activation of the IFN-induced and dsRNA-activated protein kinase PKR (Chang et al., 1992). In addition, the VV K3L protein has sequence similarity with eukaryotic initiation factor 2 α (eIF2 α) that is phosphorylated and inactivated by PKR (Beattie et al., 1991). The K3L protein competitively binds PKR and hence blocks the phosphorylation and inactivation of host eIF2 α . However, these mechanisms can only block the IFN response within infected cells, and to suppress cytokine responses more broadly, poxviruses use other strategies, such as the expression of soluble cyto-

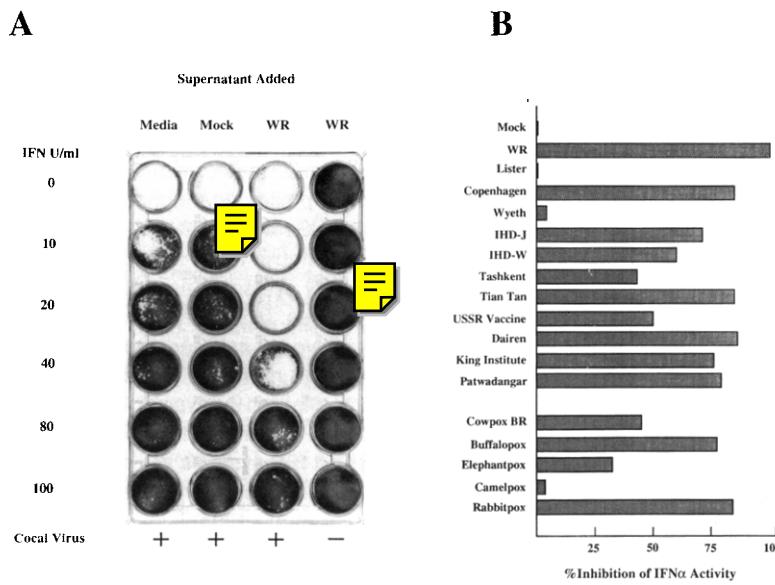


Figure 1. Supernatants from VV-Infected Cells Contain an Inhibitor of IFN α Widely Distributed in Orthopoxviruses

(A) Supernatants from VV-infected cells contain an inhibitor of IFN α . Monolayers of human HeLa cells were incubated with or without human natural IFN α at the indicated concentrations in the presence of either media alone (Media), or supernatants from 10^3 TK-143B cells either mock infected (Mock) or infected with VV strain WR (WR). After an 18 hr incubation at 37°C, the cells were challenged with 1000 pfu of cocal virus and 48 hr later stained with 0.1% crystal violet in 15% ethanol.

(B) Distribution of IFN α inhibitor in orthopoxviruses. Supernatants from 10^3 TK-143B cells infected with various VV strains or other orthopoxviruses were tested for their ability to inhibit the biological activity of 5 U of human natural IFN α . After 18 hr, the cells were challenged with 100 pfu of cocal virus and incubated for a further 48 hr in media containing 1.5% (w/v) carboxymethylcellulose before staining with 0.1% crystal violet in 15% ethanol and counting plaque numbers. The percentage inhibition of IFN α activity was calculated as described in Experimental Procedures.

kin receptors. Myxoma virus, a leporipoxvirus, expresses an IFN γ receptor that binds and inhibits rabbit IFN γ (Upton et al., 1992), and the orthopoxviruses vaccinia, cowpox, and camelpox encode a soluble IFN γ receptor of broad species specificity (Alcamí and Smith, submitted). Myxoma virus expresses a tumor necrosis factor (TNF) receptor that contributes to virus virulence (Upton et al., 1991), and Shope fibroma virus (Smith et al., 1991) and cowpox virus (Hu et al., 1994) also encode a soluble TNF receptor. VV and cowpox virus secrete an IL-1 β receptor (a member of the immunoglobulin superfamily [IgSF]) that modulates the host response to infection (Alcamí and Smith, 1992; Spriggs et al., 1992). VV gene B18R also encodes an IgSF member that is distantly related to B15R and the IL-1 and IL-6 receptors (McMahan et al., 1991; Smith and Chan, 1991) but fails to bind either cytokine (Alcamí and Smith, 1992; Spriggs et al., 1992; Morikawa and Ueda, 1993). Here, we demonstrate that B18R encodes a soluble type I IFN receptor with high affinity for human type I IFN. This receptor contributes to VV virulence, has novel broad species specificity, and belongs to a protein superfamily different from those of other type I IFN receptors.

Results

Vaccinia Virus Encodes a Soluble IFN α Inhibitor

To determine whether VV encoded a soluble inhibitor of type I IFN, virus-free supernatants from mock-infected or VV strain Western Reserve (WR)-infected cells were incubated with increasing concentrations of human natural IFN α on HeLa cells, and these cells were subsequently challenged with cocal virus, a rhabdovirus similar to vesicular stomatitis virus. Figure 1A shows that cells infected with cocal virus were lysed, but IFN α at ≥ 20 U/ml protected cells from this cytopathic effect (CPE). However, cultures

incubated with supernatants from WR-infected cells, but not mock-infected cells, developed CPE even in the presence of 40 U/ml IFN α . This CPE was not due to a VV infection, because no CPE occurred without cocal virus. Thus, WR supernatants contained an inhibitor of the IFN α subtypes present in natural human IFN α .

The IFN α Inhibitor Is Widely Distributed in Orthopoxviruses

To investigate how widely the IFN α inhibitor was distributed in orthopoxviruses, cells were infected with 14 VV strains (including rabbitpox and buffalopox), two strains of cowpox virus (Brighton red and elephantpox) and camelpox virus, and the cell supernatants were tested for inhibition of IFN α activity (Figure 1B). Inhibitory activity was found in all VV strains except Lister, but the activity was low in strain Wyeth. The lack of activity with strain Lister and the low level of activity with strain Wyeth were not due to a poor infection, as supernatants from Lister-, Wyeth-, or WR-infected cells inhibited IFN γ to a similar extent (data not shown). Cowpox, elephantpox, and camelpox also expressed an inhibitor of type I IFN, although this was at very low levels with camelpox.

Mapping of the Soluble IFN α Inhibitor

To map the VV gene encoding the soluble IFN α inhibitor, we used viruses v6/2 (Moss et al., 1981), vGS100, and vSSK2 (N. W. Blake, K. M. Law, S. Kettle, and G. L. S., unpublished data), which have large deletions in the left or right ends of the genome (Figure 2A). These regions were selected for study because other VV genes that express soluble modulators of the immune response to infection are encoded here (Smith, 1993). Supernatants from cells infected with these viruses were tested for their ability to inhibit the bioactivity of human natural IFN α or recombi-

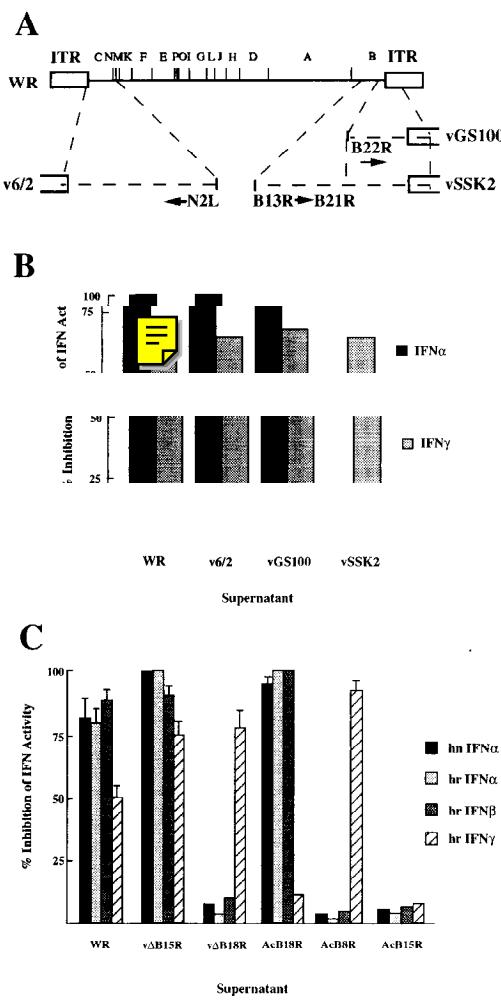


Figure 2. Mapping of the VV WR Gene Encoding the Soluble IFN- α Inhibitor to B18R

(A) HindIII restriction endonuclease map of the WR genome. The regions deleted in mutant viruses v6/2, vGS100, and vSSK2 are indicated by broken horizontal lines. The ITRs are shown boxed on the WR genome.

(B) Inhibition of IFN- α and IFN- γ activity by VV WR and mutant viruses. Monolayers of HeLa cells were incubated with supernatants from 10^3 cells infected with viruses WR, v6/2, vGS100, or vSSK2 and either 5 U of human natural IFN- α or recombinant IFN- γ for 18 hr at 37°C. The cells were then infected with approximately 100 pfu of cocal virus and incubated for a further 48 hr at 37°C in media containing 1.5% (w/v) carboxymethylcellulose. The percentage inhibition of the IFN activity was assessed as described in Experimental Procedures.

(C) Identification of B18R as the type I IFN inhibitor. Monolayers of HeLa cells were incubated with supernatants from 10^3 TK-143B cells infected with either VV WR, vΔB15R, or vΔB18R and either human natural IFN- α (hIFN- α), human recombinant IFN- α 2 (hIFN- α 2), IFN- β (hIFN- β), or IFN- γ (hIFN- γ). The IFNs were also incubated with supernatants from 10^3 Sf cells infected with recombinant baculovirus expressing B15R (AcB15R), B8R (AcB8R), or B18R (AcB18R). Experimental details were as described in (B). Bars indicate the mean \pm standard error of the mean (SEM); $n = 4$.

nant IFN- γ . Figure 2B shows that supernatants from cells infected with WR, v6/2 (a strain of VV WR with a 9 kb deletion near the left inverted terminal repeat [ITR]), or vGS100 (a strain of VV WR lacking gene B22R and all genes rightward up to the gene encoding a 7.5 kDa poly-

peptide within the right ITR) inhibited the biological activity of IFN- α . However, supernatants from vSSK2 (a strain of VV WR lacking genes B13R to B21R in addition to those missing in vGS100) did not inhibit the bioactivity of IFN- α . In contrast, all four viruses expressed a soluble IFN- γ receptor encoded by gene B8R (Alcamí and Smith, submitted). Thus, the gene encoding the IFN- α inhibitor mapped between B13R and B21R.

This region contains only two genes, B15R and B18R, predicted to encode secreted proteins (Smith and Chan, 1992; Alcamí and Smith, 1992; Spriggs et al., 1992). To determine whether B15R or B18R encoded the IFN- α inhibitor supernatants from cells infected with vi-

—, supernatants from cells infected with viruses lacking either gene, vΔB15R or vΔB18R (Alcamí and Smith, 1992), were tested for inhibition of IFN activity

but those from vΔB18R-infected cells inhibited only IFN- γ activity. This result was confirmed by using supernatants from *Spodoptera frugiperda* (Sf) insect cells infected with recombinant baculoviruses expressing either B15R or B18R (Alcamí and Smith, 1992) or B8R, a gene encoding a soluble IFN- γ receptor (Alcamí and Smith, submitted). Supernatants containing B18R (AcB18R) inhibited type I IFN, and those containing B8R (AcB8R) inhibited type II IFN, whereas B15R-containing supernatants (AcB15R) inhibited neither type of IFN. Gene B18R, therefore, encodes a type I IFN inhibitor.

The B18R Protein Blocks the Binding of IFN- α to Its Cell Surface Receptor

The biological inhibition of type I IFN mediated by B18R might occur by degradation of IFN- α or by blocking either the binding of IFN to cell receptors or some step of the IFN-responsive signaling pathway. The possible degradation of IFN was eliminated by the observed stability of 125 I-IFN- α 2 in the presence of the B18R protein (data not shown). Next, the potential of B18R to block IFN- α 2 binding to its cell receptor was investigated. Figure 3A shows that human 125 I-IFN- α 2 specifically bound to a type I IFN receptor on U937 cells, and this binding was inhibited by cold IFN- α and IFN- β , but not by IFN- γ . Similarly, supernatants from mammalian cells infected with WR and vΔB15R, but not from uninfected or vΔB18R-infected cells, and supernatants from insect cells infected with recombinant baculovirus AcB18R, but not AcB8R or AcB15R, inhibited the binding of the IFN to U937 cells. The B18R protein, therefore, blocks binding of type I IFN to its cell receptor.

The B18R Protein Binds Type I IFN

The above result suggested that the B18R protein might bind to type I IFN. This was tested in soluble binding assays with human 125 I-IFN- α 2 (Figure 3B). Supernatants from WR-infected cells bound to IFN- α 2, and the specificity of this binding was shown by its inhibition with cold human type I IFNs but not with type II IFN. B18R was confirmed as the IFN-binding protein by the binding of 125 I-IFN- α 2 to supernatants from cells infected with WR and vΔB15R but

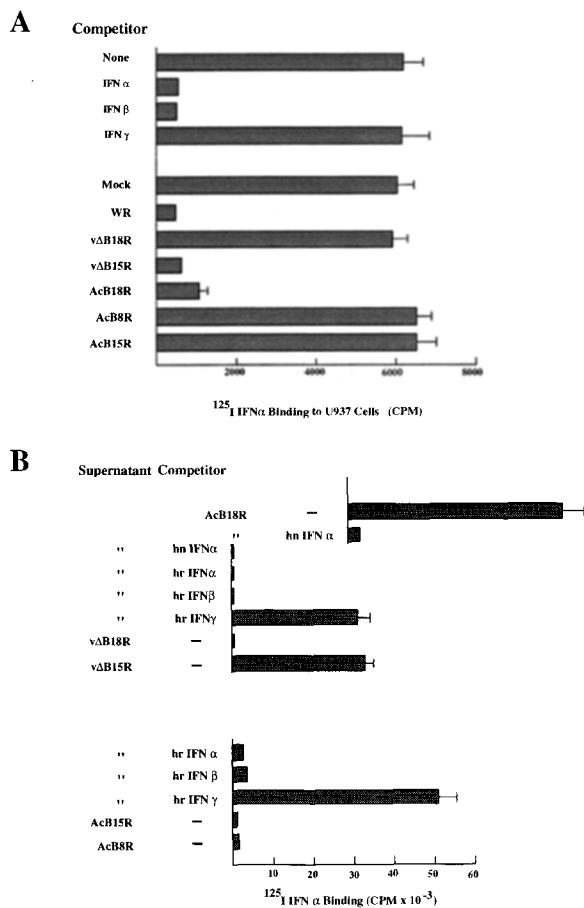


Figure 3. B18R Blocks the Binding of IFN α 2 to Its Cell Surface Receptor and Is a Soluble Receptor for Type I IFN

(A) Ten nM of ^{125}I -IFN α 2 and 10^6 U937 cells were incubated with supernatants from 10^6 WR-infected or 5×10^4 baculovirus-infected cells or with 100-fold excess of cold IFN α , IFN β , or IFN γ . After 2 hr at 4°C with constant gentle agitation, the cells were washed twice and bound and free ^{125}I -IFN α 2 separated by centrifugation through phthalate oil. Experiments were performed in duplicate tubes. Bars indicate mean \pm SEM; $n = 3$.

(B) Supernatants from 10^6 TK-143B cells infected with VV WR, vΔB15R, or vΔB18R or from 5×10^4 Sf cells infected with recombinant baculoviruses AcB15R or AcB18R were incubated at 20°C for 90 min with 50 nM human ^{125}I -IFN α 2 with or without a 100-fold excess of cold human IFN α , IFN β , or IFN γ . Receptor-bound ^{125}I -IFN was separated from free ^{125}I -IFN by filtration through GF/C glass fiber filters after addition of PEG. Experiments were performed in duplicate tubes and results expressed as specific binding after subtraction of nonspecific binding. Bars indicate mean \pm SEM; $n = 3$.

not vΔB18R. In addition, supernatants from cells infected with recombinant baculovirus AcB18R, but not AcB8R or AcB15R, specifically bound the ^{125}I -IFN α 2. These results demonstrated that B18R was a novel soluble receptor for type I IFN.

Characterization of the B18R Gene Product

Previously, the B18R gene was shown to be transcribed and translated early during infection (Ueda et al., 1990; Alcamí and Smith, 1992). This was confirmed by observing that supernatants from cells infected with VV WR in the

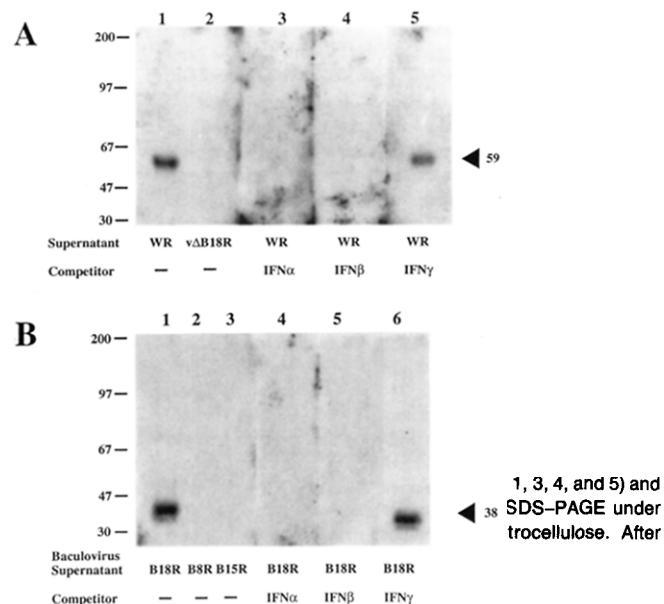


Figure 4. Ligand Blotting of B18R with ^{125}I -IFN α 2

blocking, the filters were probed with 10 ng/ml ^{125}I -IFN α 2 alone (lanes 1 and 2) or with 100-fold excess of cold competitor human recombinant IFN α 2 (lane 3), IFN β (lane 4), or IFN γ (lane 5).

(B) Supernatants from 10^6 Sf cells infected with recombinant baculovirus expressing B18R (lanes 1, 4, 5, and 6), B8R (lane 2), or B15R (lane 3) were treated as in (A). Filters were probed with 10 ng/ml ^{125}I -IFN α 2 alone (lanes 1, 2, and 3) or with 100-fold excess of cold human recombinant IFN α 2 (lane 4), IFN β (lane 5), or IFN γ (lane 6). Protein markers are in kilodaltons.

presence of cytosine arabinoside (an inhibitor of DNA synthesis and VV late gene expression) also contained an IFN α inhibitory activity (data not shown). The size of the B18R protein has been reported as either 40 kDa when translated in vitro (Ueda et al., 1990) or 60–65 kDa when secreted from VV-infected cells (Alcamí and Smith, 1992). This was further analyzed by ligand blotting (Figure 4). Supernatants from either VV- or recombinant baculovirus-infected cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, blotted onto nitrocellulose, and probed with ^{125}I -IFN α 2 with or without cold competitors. The supernatant from cells infected with WR but not vΔB18R contained a protein that bound ^{125}I -IFN α 2 and migrated at approximately 59 kDa (Figure 4A). An identically sized protein was detected after infection with all orthopoxviruses tested except VV strains Lister, Wyeth, and camelpox (data not shown). Recombinant baculovirus-derived B18R, but not B8R or B15R, also bound to ^{125}I -IFN α 2; however, the molecule migrated at 38 kDa (Figure 4B), consistent with the smaller size of the B18R protein (48 kDa) expressed from insect cells (Alcamí and Smith, 1992; Morikawa and Ueda, 1993). The binding of ^{125}I -IFN α 2 to B18R derived from either VV or AcB18R was specific, as the binding was competed with a 100-fold excess of cold type I but not type II IFN.

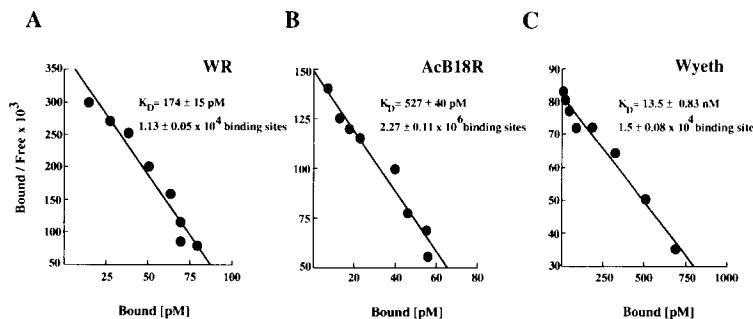


Figure 5. Scatchard Analysis of Human ^{125}I -IFN α 2 Binding to the VV IFN α/β Receptor

Supernatants from 10^6 TK-143B cells infected with WR (A), 5×10^3 Sf cells infected with AcB18R (B), or 10^6 TK-143B cells infected with VV strain Wyeth (C) were incubated in 0.2 ml with different concentrations of ^{125}I -IFN α 2 and specific bound radiolabeled ligand determined by the PEG precipitation technique. Data were converted to the Scatchard coordinate system.

Interferon Binding Characteristics of the B18R Protein

The binding affinity of B18R for human ^{125}I -IFN α 2 was assessed by Scatchard analyses (Figure 5). Supernatants from WR-, Wyeth-, and AcB18R-infected cells each showed saturation binding (data not shown). Scatchard analysis of the WR supernatant revealed single site-binding kinetics with a K_D of 174 ± 15 pM (Figure 5A) and $1.13 \times 10^4 \pm 0.05 \times 10^4$ binding sites secreted during 24 hr per infected cell. This affinity is similar to that reported for human IFN α A and the type I IFN receptor on human U937 cells (K_D , 300 pM) (Langer and Peska, 1986) and to our data (not shown) indicating 3.6×10^3 high affinity sites with a K_D of 440 pM on U937 cells. The B18R protein secreted from insect cells (Figure 5B) had a 3-fold lower affinity (K_D , 527 ± 40 pM) than the VV WR-derived protein, but the number of binding sites produced during a 72 hr infection was much greater ($2.27 \times 10^6 \pm 0.11 \times 10^6$), reflecting the high level of expression in the baculovirus system. Because VV strain Wyeth produced only low IFN α -inhibitory activity (Figure 1B), we assessed whether this was due to low receptor expression or low affinity for the ligand. Scatchard analysis (Figure 5C) revealed a similar number of binding sites per cell ($1.50 \times 10^4 \pm 0.08 \times 10^4$) compared with the number for WR, but with a K_D of only 13.5 ± 0.83 nM, 78-fold lower than the WR B18R protein.

Species Specificity of the VV Type I IFN Receptor

The relative binding affinities of B18R for type I IFN from different species were compared by competition binding of unlabeled IFNs with human ^{125}I -IFN α 2. Equilibrium dissociation constants were calculated from the EC_{50} values using the Cheng-Prusoff relationship (Cheng and Prusoff, 1973). These calculations were based on a K_D of 174 pM for the binding of human ^{125}I -IFN α 2 to the WR B18R protein (Figure 5A) and could only be performed where the competitor contained only one subtype of IFN. The competitive binding curves (Figure 6A) revealed that type I IFN from different species bound to the WR B18R protein with varying affinities. Human and rabbit type I IFN bound to B18R with indistinguishable high affinity, and bovine and rat IFN α bound with reduced affinity, with an estimated K_D of 1 nM, whereas mouse type I IFNs bound with low affinity, with an estimated K_D of 27 nM for mouse IFN β .

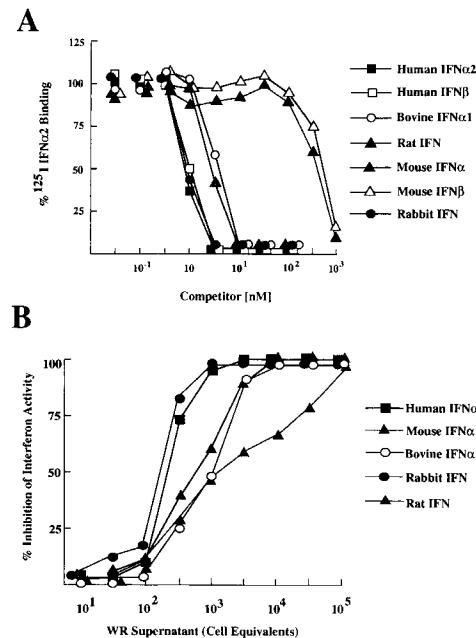


Figure 6. Species Specificity of B18R Binding and Inhibition of Type I IFN

(A) Cross-competition of human ^{125}I -IFN α 2 binding to the supernatant from 10^6 WR-infected cells. ^{125}I -IFN α 2 (10 nM) was incubated with soluble VV WR IFN type I receptor together with increasing concentrations of cold human recombinant IFN α 2 (closed squares), human recombinant IFN β (open squares), bovine recombinant IFN α 1 (open circles), natural rat type I IFN (shaded triangles), mouse natural IFN α (closed triangles), mouse natural IFN β (open triangles), and natural rabbit type I IFN (shaded circles) for 2 hr at 20°C. Ligand-receptor complexes were separated from free ligand by precipitation with PEG. The concentrations of the cold IFNs were calculated from their known biological activities per microgram of protein and are expressed as the concentrations of active IFN.

(B) Various concentrations of supernatant from WR-infected cells were incubated with 5 U of either human natural IFN α (closed squares), mouse natural IFN α (closed triangles), bovine recombinant IFN α 1 (open circles), or natural rabbit (shaded circles) or natural rat type I IFN (shaded triangles) with cells of the appropriate species (human, HeLa cells; mouse, L929 cells; bovine, MDBK cells; rabbit, RK13 cells; and rat, NRK cells). After 18 hr, 100 pfu of cocal virus were added and the culture incubated for a further 48 hr, before staining with crystal violet and counting plaques. The percentage inhibition of the IFN activity was calculated as described in the Experimental Procedures.

Table 1. Effect of B18R Deletion on Vaccinia Virus Virulence in Mice

Dose (pfu)	WR	vΔB18R	vB18R-R
10 ⁴	5 of 5	0 of 5	2 of 5
10 ⁵	5 of 5	0 of 5	5 of 5
10 ⁶	5 of 5	5 of 5	5 of 5
10 ⁷	5 of 5	3 of 5	5 of 5
10 ⁸	5 of 5	5 of 5	5 of 5

Groups of five female, 5- to 6-week-old BALB/c mice were infected intranasally with the indicated doses of virus. Mortalities, including mice that had been sacrificed owing to severe infection and loss of >30% of body weight, that had occurred by day 14 after infection are shown.

To analyze further the species specificity of the WR type I IFN receptor, the ability of supernatant from WR-infected cells to inhibit type I IFN bioactivity from a number of different species was examined. Figure 6B shows that B18R inhibits type I IFN activity from all species tested, but in agreement with the binding data, human and rabbit IFN were inhibited most effectively, with supernatant from only 10³ cells causing complete inhibition of 5 U of IFN. Rat and bovine IFN were inhibited less efficiently, requiring supernatants from 10⁴ cells to inhibit the activity of 5 U of IFN, and the supernatant from 10⁵ cells was required to cause complete inhibition of 5 U of mouse IFN. In all cases, supernatants from vΔB18R-infected cells tested at the same concentrations mediated no inhibition of the IFN activity (data not shown).

Virulence of VV Lacking B18R in Mice

The contribution of B18R to VV virulence was tested in a murine intranasal model (Turner, 1967; Williamson et al., 1990) in which at doses of $\geq 10^4$ plaque-forming units (pfu) of VV WR, there is an extensive respiratory infection, followed by virus dissemination and death. To be certain that any difference observed in the phenotype of WR and vΔB18R was due only to loss of B18R, a virus revertant, vB18R-R, was constructed by transient dominant selection (Falkner and Moss, 1990; Isaacs et al., 1990) in which the B18R gene was reinserted into the endogenous locus of the vΔB18R genome. The viruses vΔB18R and vB18R-R did not contain the selective marker that might affect their phenotype. The correct structure of the B18R gene and flanking genes in vB18R-R was confirmed by Southern blot hybridization and polymerase chain reaction (PCR) as described in Experimental Procedures (data not shown), and the expression of an active B18R gene was confirmed by binding assays with human ¹²⁵I-IFN α 2 (data not shown).

Table 1 shows that deletion of gene B18R reduced VV virulence. At doses of 10⁴ and 10⁵ pfu, most mice infected with WR or vB18R-R were sacrificed, owing to a severe infection and the loss of $\geq 30\%$ of body weight, while vΔB18R-infected animals survived. The attenuation of the virus was most clearly illustrated at a dose of 10⁴ pfu, where mice infected with vΔB18R showed no sign of ill-

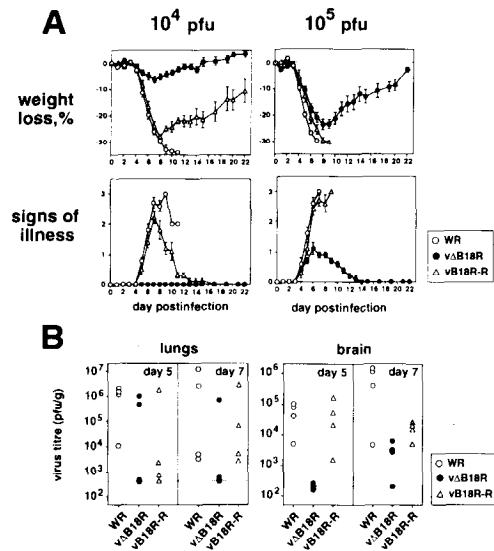


Figure 7. Virulence of vΔB18R in Mice

(A) Groups of five mice were intranasally infected with 10⁴ or 10⁵ pfu of WR (open circles), vΔB18R (closed circles), or vB18R-R (open triangles). Mice were individually weighed and monitored for signs of illness, scored from zero to four, daily. The mean percentage weight loss of each group \pm SEM, compared with the weight immediately prior to infection, and the mean value of signs of illness \pm SEM in each group are shown. All mice in the groups infected with WR (10⁴ and 10⁵ pfu) or vB18R-R (10⁵ pfu) and two mice infected with 10⁴ pfu of vB18R-R died or were sacrificed. None of the vΔB18R-infected mice were sacrificed.

(B) Mice were intranasally infected with 10⁴ pfu of vaccinia virus WR (open circles), vΔB18R (closed circles), or vB18R-R (open triangles). On the indicated days after infection, four animals infected with each virus were sacrificed and the titer of infectious virus in the lungs and the brain determined by plaque titration in BS-C-1 cell monolayers. Virus titers are expressed as plaque-forming units per gram of tissue. The minimum detection limit of the plaque assay is indicated with a broken line.

ness (ruffled fur, arched backs, or reduced mobility) and lost only a maximum of 5% of body weight (Figure 7A). In contrast, animals infected with WR or vB18R-R showed severe signs of illness and weight loss, and many were either sacrificed or died. Similarly, infection with 10⁵ pfu was very severe with WR and vB18R-R (all animals died or were sacrificed), and although all vΔB18R-infected animals showed signs of illness and weight loss, they recovered.

The replication of VV in the lungs and brains of mice on days 5 and 7 after infection was also investigated (Figure 7B). Only three out of eight vΔB18R-infected mice had detectable virus in the lungs. In contrast, all eight mice infected with WR and seven out of eight mice infected with vB18R-R yielded virus and yielded it at higher titers than did the vΔB18R-infected animals. Dissemination of vΔB18R within the infected animal was even more restricted, and on day 5, no virus was detected in the brain, whereas all mice infected with WR or vB18R-R yielded virus. The type I IFN receptor, therefore, contributes to VV virulence in this model.

Discussion

This study demonstrates that most orthopoxviruses express a soluble receptor for type I IFNs, which in VV strain WR is encoded by gene B18R. This gene was predicted to encode a secretory IgSF glycoprotein with similarity to another VV IgSF protein, B15R, and to the IL-1 and IL-6 receptors (McMahan et al., 1991; Smith and Chan, 1991). The B15R protein was later shown to be a 50–60 kDa soluble IL-1 β receptor (Alcamí and Smith, 1992; Spriggs et al., 1992), while B18R encodes a 60–65 kDa glycoprotein that is expressed on the cell surface and is secreted from the cell (Ueda et al., 1990; Alcamí and Smith, 1992; Morikawa and Ueda, 1993). Although B18R has 20% amino acid identity with B15R, it does not bind IL-1 or IL-6, and its ligand had hitherto not been identified (Alcamí and Smith, 1992; Morikawa and Ueda, 1993). B18R was known, however, to encode the S antigen that is expressed by most orthopoxviruses, but not VV strain Lister (Ueda et al., 1969; Ueda et al., 1972; Amano et al., 1979; Ueda et al., 1990; Morikawa and Ueda, 1993). It is surprising that B18R is a type I IFN receptor, as it is an IgSF member, while other type I IFN receptors are CRF2 members (Cal-lard and Gearing, 1994).

Characterization of the VV WR type I IFN receptor showed that the protein is expressed early during infection and has higher affinity for human IFN α and IFN β (K_D , 174 pM) than do the human type I IFN receptors (200 pM to 5 nM). This is consistent with the VV WR type I IFN receptor being an effective competitor for IFN binding in vivo. Cells infected with VV secreted 1.1×10^4 type I IFN receptors per cell, lower than the number of IL-1 β receptors (1×10^5 per cell) produced by the same virus (Alcamí and Smith, 1992). This difference may be partly attributable to expression from an early (B18R) rather than a late (B15R) promoter, but may also reflect the retention of some B18R on the cell surface (Ueda et al., 1990; Alcamí and Smith, 1992; Morikawa and Ueda, 1993). Whether B18R present on the cell surface can still bind type I IFN is under investigation.

The affinity of the baculovirus-derived B18R was 3-fold lower than that of the VV WR B18R, and, consistent with its higher affinity, VV WR B18R inhibited IFN binding to U937 cells more effectively than did baculovirus-derived B18R. This lower affinity may be due to the incomplete glycosylation of the B18R protein from insect cells. The sizes of the B18R protein derived from insect and mammalian cells (38 and 59 kDa, respectively) detected by ligand blotting under nonreducing conditions (Figure 4) were broadly consistent with the sizes observed from these cells (48 and 60–65 kDa, respectively) by immunoprecipitation and electrophoresis under reducing conditions (Alcamí and Smith, 1992). The different running conditions may explain the different sizes observed. The size of the B18R type I IFN receptor was shown by ligand blotting to be highly conserved in orthopoxviruses (data not shown).

The VV strain Wyeth type I IFN receptor has 78-fold lower affinity for human IFN α than does the WR-derived protein, while the proteins were expressed at similar lev-

els. The sequence of the B18R gene in VV Wyeth will, therefore, be of interest. The lack of expression of a type I IFN receptor by VV Lister was consistent with the previous observation that this virus does not express the S antigen (Amano et al., 1979), which was shown to be encoded by gene B18R (Ueda et al., 1990). It is also noteworthy that Wyeth, which expresses a low affinity receptor, and Lister, which expresses no receptor, were widely used smallpox vaccines that gave lower frequencies of postvaccinal neurological complications than many other strains (Fenner et al., 1988). Possibly the lack of a high affinity soluble receptor for human type I IFN contributed to the relative safety of these vaccine strains.

With the exception of the binding of human type I IFN to bovine IFN receptors, the type I IFNs demonstrate a strict species specificity even to the degree that mouse and rat IFNs cross-react poorly. The broad species specificity of the type I IFN receptor from VV is therefore remarkable. This might be because the VV receptor is an IgSF rather than a CRF2 member, and this may permit greater structural flexibility to confer the broad specificity. The VV type I IFN receptor has a very high affinity for human IFN α and IFN β (K_D , 174 pM) and rabbit type I IFN, a high affinity for bovine (K_D , 1 nM) and rat type I IFN, and an affinity for mouse IFN β (K_D , 27 nM) 150-fold lower than that for human IFN α . Nevertheless, this affinity is still sufficient for the receptor to bind and inhibit mouse IFN α , and, consonant with this, the virus lacking the B18R gene was attenuated in intranasally infected mice.

The relative affinities of the VV type I IFN receptor for type I IFN from different species are relevant to the enigmatic origin of VV and which host(s) this virus might have naturally infected during evolution. Jenner introduced cowpox for vaccination against smallpox in 1798, but the smallpox vaccines used this century are VV, not cowpox (Downie, 1939). Thus, sometime between 1798 and 1939, VV replaced cowpox as the smallpox vaccine. Molecular analyses of the genomes of cowpox, VV, and variola (Mackett and Archard, 1979; Goebel et al., 1990; Smith et al., 1991; Aguado et al., 1992; Shchelkunov et al., 1993; Massung et al., 1994) have shown that VV is unlikely to have arisen by mutation from cowpox or variola or by recombination between these viruses, and a favored hypothesis for the origin of VV is that it is an independent orthopoxvirus that previously infected a species in which it is no longer endemic. Horsepox has been suggested (Baxby, 1981), as early vaccinators were reported to obtain supplies of vaccine from poxvirus infections of horses when supplies from infected cows were scarce. The affinities of the VV type I IFN receptor for IFN from different species indicate that the mouse is less likely than human, rabbit, cow, or rat to have been a natural host. Consistent with this, an analysis of the species specificity of the VV and cowpox IFN γ receptor showed that these bound and inhibited the activity of IFN γ from human, cow, and rat, but not mouse (Alcamí and Smith, submitted).

The importance of IFN for protection against virus infection is illustrated by the consequences of direct IFN treatment and the inactivation of components of the IFN sys-

tem, but also by the numerous measures taken by viruses to counteract IFN (Introduction). Many viruses, including VV, achieve resistance to IFN by targeting the intracellular IFN-induced enzyme PKR (for review, see Smith, 1994). But poxviruses also present an extracellular blockade of type II (Upton et al., 1992; Alcamí and Smith, submitted) and type I (this paper) IFNs. The latter represents a novel mechanism for VV inhibition of type I IFN, the fourth VV protein that interferes with IFN and the fourth soluble cytokine receptor identified in poxviruses.

Deletion of the poxvirus cytokine receptors has variable effects on virus virulence. Loss of the TNF receptor from myxoma virus attenuated the virus in rabbits (Upton et al., 1991). Inactivation of the IL-1 β receptor from VV caused a 100-fold increase in LD₅₀ when inoculated intracranially into mice (Spriggs et al., 1992). In contrast, intranasal infection, a more physiologically relevant route, with v Δ B15R caused an increase in virulence (Alcamí and Smith, 1992). The effect of deletion of the IFN γ receptor on virulence is unknown. Here, deletion of the IFN α/β receptor from VV WR is shown to attenuate VV in a murine intranasal model and to diminish virus replication in the lungs and dissemination to the brain. The degree of attenuation observed in this model is modest compared with that resulting from deletion of structural proteins of the envelope of extracellular enveloped virus (Engelstad and Smith, 1993; Parkinson and Smith, 1994). This may reflect the relatively low affinity of the VV type I IFN receptor for mouse type I IFN, and the retention by the B18R deletion mutant of other genes (E3L and K3L) that confer resistance to IFN. The VV type I IFN receptor shares 89% amino acid identity with an open reading frame (ORF) in variola major virus strains India-1967 and Bangladesh-1975 (Massung et al., 1993, 1994; Shchelkunov et al., 1993). Such a high degree of amino acid identity makes it probable that the variola protein would bind IFN with similar species specificity and would have contributed to variola virus virulence in humans.

Other soluble cytokine receptors expressed by poxviruses (for TNF, IL-1 β , and IFN γ) were recognized only after the sequencing of mammalian homologs, owing to their amino acid identity (24%–38%) with the extracellular ligand-binding domains of the cellular receptors. In contrast, no mammalian homolog has been described for the VV type I IFN receptor. Preliminary analysis does not show any significant sequence similarity of B18R with the extracellular domain of the known human IFN α/β receptors. However, the similar folding pattern of fibronectin type III and immunoglobulin domains (Barclay et al., 1993) may facilitate the interaction of two unrelated receptors with type I IFNs. If B18R has been acquired from the host, as seems likely for other poxvirus cytokine receptors, the sequence of the B18R gene may aid the cloning of a cellular counterpart. Such a host protein might be a soluble inhibitor of IFN action or a cell surface agonist or antagonist. Although an analysis of human serum IFN-binding proteins only identified a soluble form of the 51 kDa IFN type I receptor (Novick et al., 1992, 1994), cross-linking of radiolabeled IFN α to cell surfaces identified multiple complexes (Colamonici et al., 1992).

In summary, we have shown that the orthopoxviruses encode a high affinity IFN α/β receptor of novel structure and broad species specificity. The presence of an IFN α/β receptor in the orthopoxvirus genome adds to the impressive array of anti-IFN mechanisms employed by the virus and emphasizes the importance of IFN in defense against viruses. Lastly, this study illustrates that the analysis of virus immune evasion mechanisms may reveal potentially novel components of the mammalian immune system as well as imparting fundamental information about virus pathogenesis.

Experimental Procedures

Cells and Viruses

Human U937, HeLa, and D980R cells, mouse L929 cells, bovine MDBK cells, rat NRK cells, and rabbit RK13 cells were obtained from the Cell Bank of the Sir William Dunn School of Pathology (University of Oxford). Human TK-143B cells were obtained from the American Type Culture Collection. Cells were grown in minimal essential medium (MEM) (GIBCO) with 10% fetal bovine serum (FBS), except U937 cells, for which RPMI 1640 (GIBCO) and 10% FBS were used. Sf 21 insect cells and AcNPV were obtained from R. Posse (Natural Environment Research Council Institute of Virology and Environmental Microbiology, Oxford) and were cultured in TC100 medium (GIBCO) containing 10% FBS. The sources of VV strains and other orthopoxviruses have been described (Alcamí and Smith, 1992; Alcamí and Smith, submitted). Cocal virus was obtained from W. James (Sir William Dunn School of Pathology, University of Oxford).

Cytokines

Human recombinant IFN α 2 (specific activity, 3×10^8 U/mg) was obtained from Proterec (London, England) and was radioiodinated without loss of biological activity by using 125 I Bolton–Hunter reagent (specific activity, 2000 Ci/mmol; Amersham, England) to a specific activity of 40 μ Ci/ μ g (Langer and Peska, 1986). Human natural IFN α (specific activity, 1.5×10^8 U/mg) derived from Namalwa cells was obtained from Wellcome (Beckenham, England), human recombinant IFN β (specific activity, 2×10^8 U/mg) from GIBCO BRL, mouse IFN α and IFN β (specific activity, 4×10^4 U/mg and 1.3×10^6 U/mg, respectively) from Calbiochem, and recombinant human and mouse IFN γ (1×10^7 U/mg) from Genzyme; bovine recombinant IFN α 1 (specific activity, 1×10^7 U/mg) was a generous gift from R. A. Collins (Institute of Animal Health, Compton, England); and rat and rabbit type I interferon (specific activity, 1.9×10^6 U/mg and 3.9×10^5 U/mg, respectively) were from Lee Biomolecular Research Laboratories, Incorporated (San Diego, California).

Preparation of Supernatants from Virus-Infected Cells

Cultures of TK-143B cells or Sf cells were infected with 10 pfu/cell. Supernatants from orthopoxvirus- or baculovirus-infected cells were harvested 3 days postinfection and centrifuged at 3000 rpm for 10 min at 4°C and the pellet discarded. Virus particles were removed by centrifugation at 16,500 rpm in a SW41 Ti rotor for 60 min at 4°C. The supernatants were then concentrated to that equivalent to 3×10^7 cells/ml in Centriprep-10 concentrators (10,000 MW cutoff; Amicon) and stored at –20°C.

Biological Assays for IFN

The biological activity of IFN from different species was assayed by its ability to inhibit cocal virus plaque formation (Alcamí and Smith, submitted). Human, mouse, bovine, rat, and rabbit IFN were assayed in cultures of human HeLa cells, mouse L929 cells, bovine MDBK cells, rat NRK cells, and rabbit RK13 cells, respectively. Cell monolayers in 24-well plates (Nunc) were pretreated in 0.5 ml of MEM containing 10% FBS and the indicated doses of IFN and supernatants from orthopoxvirus- or recombinant baculovirus-infected cultures. After an 1 hr incubation at 37°C, the cells were infected with approximately 100 pfu of cocal virus and the cells incubated for a further 48 hr at 37°C in media containing 1.5% (w/v) carboxymethylcellulose. The percentage

inhibition of IFN activity was calculated by dividing the number of viral plaques in the wells containing viral supernatant and IFN by the number of plaques in the wells receiving cocal virus alone. Supernatants from the infected cultures did not form plaques in the absence of cocal virus.

Cell Surface and Soluble IFN Receptor Binding Assays

For cell surface receptor binding assays, cells were harvested and washed twice in ice-cold binding medium (RPMI 1640 containing 1% [w/v] bovine serum albumin, 0.1% [w/v] NaN_3 , and 20 mM HEPES [pH 7.2]). Cells (10^6) were incubated in duplicate with ^{125}I -IFN α 2 in 0.2 ml of binding buffer alone or containing 100-fold excess of unlabeled IFN for 2 hr at 4°C with constant gentle agitation. The cells were subsequently washed twice in ice-cold protein-free binding buffer and resuspended in 0.1 ml of the same buffer. Bound and free ^{125}I -IFN α 2 were separated by phthalate oil centrifugation (Dower et al., 1985) and the tube tips excised and radioactivity counted in a gamma counter.

Soluble receptor binding assays were performed in duplicate with supernatants from VV- or baculovirus-infected cells by incubation with ^{125}I -IFN α 2 in a final volume of 0.2 ml for 90 min at room temperature. The ligand receptor complexes were precipitated with polyethylene glycol (PEG) and the precipitate collected on Whatman GF/C filters as described (Symons et al., 1990). Nonspecific binding of ^{125}I -IFN α 2 precipitated with binding medium alone was subtracted. Binding data were analyzed with the LIGAND program (Munson and Rodbard, 1980).

Ligand Blotting

Supernatants from 10^5 VV- or 10^4 baculovirus-infected cells were resolved by SDS-PAGE under nonreducing conditions on a 7.5% gel and transferred onto 0.45 μm nitrocellulose (Schleicher and Schuell). After the filters were blocked in 3% (w/v) nonfat skimmed milk in 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 0.02% (w/v) NaN_3 overnight at 4°C, the filters were probed with 10 ng/ml ^{125}I -IFN α 2 in blocking buffer with or without 1.0 $\mu\text{g}/\text{ml}$ cold IFN for 4 hr at room temperature. Blots were then washed three times for 30 min in blocking buffer, air-dried, and exposed to autoradiography film.

Construction of Recombinant Vaccinia Viruses

The VV WR B18R deletion mutant, v Δ B18R (Alcamí and Smith, 1992), was used for the construction of a revertant virus containing the B18R gene in its endogenous locus by transient dominant selection (Falkner and Moss, 1990; Isaacs et al., 1990). A plasmid, pAA21, was constructed in which a BamHI-XbaI DNA fragment, excised from pAA2 (Alcamí and Smith, 1992) and containing the entire B18R gene and 380 and 890 nt of the 5' and 3' flanking regions, respectively, was cloned into BamHI- and XbaI-cut pSJH7 (Hughes et al., 1991). This plasmid was transfected into cells infected with v Δ B18R, and after isolation of mycophenolic acid-resistant intermediate viruses, a revertant was isolated on D980R cells (Kerr and Smith, 1991) by using 6-thioguanine (Isaacs et al., 1990). The virus revertant isolated was termed vAA15 and is referred to here as vB18R-R.

The genomes of v Δ B18R and vB18R-R were analyzed by using virus DNA extracted from virus cores (Esposito et al., 1981). Viral DNA was cut with three different enzymes and analyzed by Southern blotting using fluorescein-labeled probes specific for B18R and the right hand end of the genome. PCR analyses using oligonucleotides flanking the B16L, B17R, B18R, B19R, and B20R ORFs of VV WR were also performed. These analyses confirmed that the genomic structure of each virus was as expected and that no other alterations had occurred at the right hand end of the virus genome (data not shown).

Recombinant Baculovirus

The recombinant baculoviruses expressing B15R (AcAA3), B18R (AcAA4), and B8R (AcAA5) are described elsewhere (Alcamí and Smith, 1992; Alcamí and Smith, submitted) and are referred to here as AcB15R, AcB18R, and AcB8R, respectively.

Assay of Virus Virulence

Female BALB/c mice (5–6 weeks old) were anesthetized and infected intranasally with 20 μl of the diluted virus in 0.5% bovine serum albumin in phosphate-buffered saline (Turner, 1967; Williamson et al.,

1990). Each day, mice were individually weighed and monitored for signs of illness, and those suffering a severe infection and having lost $\geq 30\%$ of body weight were sacrificed. To determine virus titers in organs, mice were sacrificed 5 or 7 days postinfection and their lungs and brains removed, weighed, homogenized in 1 ml of phosphate-buffered saline, frozen and thawed three times, and sonicated. Virus infectivity was determined by plaque assay on BS-C-1 cells.

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