The vaccinia virus soluble interferon-γ receptor is a homodimer

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The vaccinia virus (VV) interferon (IFN)-γ receptor (IFN-γR) is a 43 kDa soluble glycoprotein that is secreted from infected cells early during infection. Here we demonstrate that the IFN-γR from VV, cowpox virus and camelpox virus exists naturally as a homodimer, whereas the cellular IFN-γR dimerizes only upon binding the homodimeric IFN-γ. The existence of the virus protein as a dimer in the absence of ligand may provide an advantage to the virus in efficient binding and inhibition of IFN-γ in solution.

Vaccinia virus (VV) is an orthopoxvirus that expresses many proteins that modulate the host response to infection (Smith et al., 1997b). One strategy to suppress the host response to infection employed by VV and other poxviruses is to express proteins that are secreted from the infected cell and which bind host cytokines, chemokines, complement factors or interferons (IFNs). These secreted virus proteins often share amino acid similarity with host proteins that bind the same ligands, although this is not always the case. To date, VV has been shown to express soluble proteins that bind tumour necrosis factor (TNF) (Alcamí et al., 1999), interleukin (IL)-1β (Alcamí & Smith, 1992; Spriggs et al., 1992), type I IFNs (Colamonici et al., 1995; Symons et al., 1995), IFN-γ (Alcamí & Smith, 1995; Mossman et al., 1995a), complement factor C3b and C4b (Kotwal et al., 1990), IL-18 (Smith et al., 2000) and a wide range of CC-chemokines (Graham et al., 1997; Smith et al., 1997a; Alcamí et al., 1998). Most of these virus immunomodulators are present only in solution where they bind and inhibit the host factor; however, the IFN-α/β receptor encoded by gene B18R of VV strain Western Reserve is also present on the cell surface where it can protect cells against the antiviral effects of IFN (Alcamí et al., 2000). Three strains of VV also express a TNF receptor that is present on the cell surface (Alcamí et al., 1999). This paper concerns the IFN-γ receptor (IFN-γR) encoded by gene B8R of VV strain WR.

IFNs are soluble species-specific glycoproteins that bind to receptors on cells and trigger signal transduction leading to the expression of IFN-responsive genes and the establishment of an antiviral state (Samuel, 1991; Goodbourn et al., 2000). Subsequent virus infection activates pathways leading to the inhibition of protein synthesis and hence inhibition of virus replication. IFNs are grouped according to the type of receptor to which they bind. Type I IFNs (IFN-α and -β) bind to a common receptor called the type I IFN receptor, whereas type II IFN (IFN-γ) binds to the type II IFN receptor (IFN-γR). The IFN-γR is an 85–90 kDa type I membrane glycoprotein and belongs to the type II cytokine receptor family (Aguet et al., 1988; Farrar & Schreiber, 1993). It is present on the surface of cells as a monomer but dimerizes upon binding IFN-γ (Fountoulakis et al., 1991, 1992; Greenlund et al., 1993) leading to signal transduction. IFN-γ is a 17 kDa α-helical glycoprotein that exists as a non-covalent homodimer and its crystal structure has been determined alone (Ealick et al., 1991;Samudzi et al., 1991) and complexed with its receptor (Walter et al., 1995).

Poxvirus-encoded IFN-γRs were identified first in myxoma virus: the secreted T7 protein (M-T7) bound and inhibited the biological activity of rabbit IFN-γ (Upton et al., 1992; Mossman et al., 1995b) and was important for myxoma virus virulence in rabbits (Mossman et al., 1996). Computational analyses showed that VV (Howard et al., 1991), other orthopoxviruses (Massung et al., 1994; Shchelkunov et al., 1998), capripoxvirus (Cao et al., 1995) and swinepox (Massung et al., 1993) encoded related proteins. Functional studies showed that the VV, cowpox virus, camelpox virus and ectromelia virus IFN-γRs were secreted from infected cells, bound IFN-γ from a wide range of species and prevented IFN-γ from binding to its cellular receptor (Alcamí & Smith, 1995; Mossman et al., 1995a). The broad species-specificity of the IFN-γR encoded by these orthopoxviruses was notable because cellular IFN-γRs are usually species-specific. A surprising feature of the VV protein was its inability to inhibit mouse IFN-γ although it did inhibit rat, rabbit, cow, human (Alcamí & Smith, 1995; Mossman et al., 1995a) and chicken (Puehler et al., 1998) IFN-γ. Nevertheless, a
VV mutant lacking the B8R gene encoding the IFN-γR was attenuated in a mouse model (Verardi et al., 2001).

Here we have studied the physical state of the VV IFN-γR and report that the protein exists naturally in solution as a homodimer. Hitherto, the size of the VV IFN-γR expressed by VV strain Western Reserve (WR) or by recombinant baculovirus (AcB8R) was investigated by studying the size of the complex of the virus protein chemically cross-linked with human IFN-γ after electrophoresis on SDS-polyacrylamide gels (Alcamí & Smith, 1995; Mossman et al., 1995a). These studies suggested that the protein from mammalian cells had a size of 43 kDa and when secreted from insect cells was 32–35 kDa. The difference was attributed to the different pattern of glycosylation in mammalian and insect cells. To investigate the size of the protein further and analyse the nature of the complex formed with IFN-γ, supernatants from cells infected with VV or recombinant baculovirus were run on polyacrylamide gels in the absence of reducing agent, blotted to nitrocellulose filters and probed with human 125I-IFN-γ. Under these conditions 125I-IFN-γ bound to proteins with an electrophoretic mobility that indicated a size between 75 and 80 kDa (data not shown). This observation suggested that the VV 125I-IFN-γR might be a dimer and this was investigated further.

Fig. 1 shows that when the supernatant from VV strain WR-infected cells was incubated with human 125I-IFN-γ and protein-complexes were cross-linked chemically with EDC as described (Alcamí & Smith, 1995) and analysed by SDS–PAGE in the presence of 5% β-mercaptoethanol, three labelled bands were detected. The smaller structures (approximately 17 and 35 kDa) represent monomeric and dimeric 125I-IFN-γ respectively, whereas the larger structure (about 60 kDa) represents a complex containing one molecule of the VV IFN-γR and a monomer of 125I-IFN-γ, as reported previously (Alcamí & Smith, 1995). However, when the chemically cross-linked samples were analysed by SDS–PAGE in the absence of reducing agent, the 60 kDa complex was absent and was replaced by a higher molecular mass complex that had a size consistent with it containing two molecules of the VV IFN-γR and two monomers of 125I-IFN-γ. Similar size complexes were formed with the IFN-γR expressed by cowpox virus and camelpox virus, indicating this seemed to be a general feature of orthopoxvirus IFN-γRs, rather than being specific to VV. No complexes were detected when the supernatants from mock-infected cells were analysed in parallel.

The observation that the VV IFN-γR was detected as a complex with two molecules of IFN-γR and two monomers of 125I-IFN-γ did not establish whether the virus protein existed naturally as a dimer or whether dimerization occurred only after binding the dimeric IFN-γ, which is known to induce dimerization of IFN-γR on cells. To address this, the VV IFN-γR expressed by recombinant baculovirus (AcB8R) was analysed by SDS–PAGE in the presence or absence of β-mercaptoethanol and was compared to the VV IL-1βR and IFN-α/βR expressed in the same way (Fig. 2a). SF21 cells were infected with the recombinant baculoviruses AcB8R (Alcamí & Smith, 1995), AcB15R or AcB18R (Alcamí & Smith, 1992; Symons et al., 1995) and were labelled with [35S]methionine and [3H]cysteine from 24–48 h post-infection (p.i.). Proteins in the cell culture supernatant were analysed by SDS–PAGE and autoradiography. The complexes that are represented by the different bands on the gel are illustrated by filled circles for IFN-γ subunits and shaded ovals for IFN-γR subunits. Size markers are shown in kDa.

![Fig. 1. Cross-linking of IFN-γ to IFN-γR from VV, cowpox virus and camelpox virus forms a high molecular mass complex.](image-url)
Fig. 2. The VV IFN-γR exists as a dimer in the absence of IFN-γ. (a) SDS–PAGE in the presence or absence of β-mercaptoethanol. Insect cells were infected with the indicated baculoviruses at 10 p.f.u. per cell and labelled with [35S]methionine and [35S]cysteine from 24–48 h p.i. Cell culture media were then harvested, centrifuged to pellet detached cells and debris and the proteins in the supernatant was analysed by SDS–PAGE in the presence or absence of β-mercaptoethanol (ME), as indicated, followed by fluorography. The positions of the VV IL-1βR and IFN-α/βR (open arrowheads), the VV IFN-γR (closed arrowheads) and molecular size markers are indicated in kDa. (b) Sucrose density gradient centrifugation. 35S-labelled IFN-γR was prepared as in (a) and layered onto a 12 ml 5–25% (w/v) continuous sucrose gradient in PBS. The gradient was centrifuged in a Beckman SW41 Ti rotor at 40000 r.p.m. for 36 h at 4 °C before fractions were collected. Fractions were mixed with 5 µg of BSA and proteins were precipitated with 10% trichlorotetraacetic acid (for 10 min at 4 °C) and collected by centrifugation (for 10 min in a microfuge). Pulses were dissolved in SDS–PAGE sample buffer, their pH was adjusted by addition of ammonium vapours and they were analysed by scintillation counting and plotted (graph) or by SDS–PAGE and fluorography. Prestained molecular size markers were analysed in parallel and the sucrose concentration in the fractions was confirmed to be similar by determination of the refractive index. The positions of molecular size markers (in kDa) are shown. The original sample (B8R) was also analysed by SDS–PAGE.

The ability of the IFN-γR to form dimers was investigated further by density gradient centrifugation (Fig. 2b). Soluble 35S-labelled protein produced from AcB8R-infected insect cells was applied to a sucrose density gradient and centrifuged for 36 h at 40000 r.p.m. at 4 °C. The gradient was fractionated and the insoluble radioactivity in each fraction was determined by scintillation counting (graph) and the proteins were analysed by SDS–PAGE and fluorography. Fractions 4 to 6 contained the majority of the insoluble radioactivity and the VV IFN-γR. These fractions corresponded to a protein of approximately 68 kDa, representing a dimer of the 32–35 kDa B8R protein expressed from recombinant baculovirus. Both these analyses indicated that the virus protein was a homo-dimer in the absence of ligand.

The nature of the interaction between the monomeric subunits was investigated next. Soluble 35S-labelled protein produced from AcB8R-infected insect cells was mixed with Laemmli buffer that did or did not have reducing agent and was analysed by SDS–PAGE. As noted previously, the protein ran as either a monomer or dimer in the presence or absence of reducing agents, respectively (Fig. 3a). To determine which conditions were able to disrupt the dimer, the protein was mixed with Laemmli buffer without reducing agent and the effect of salt or β-mercaptoethanol concentration, and pH were assessed (Fig. 3b). Increasing the salt concentration or pH failed to disrupt the complex, and addition of reducing agent destroyed the complex completely. Collectively, these data suggest that disulphide bonds are needed to maintain the correct conformation of the complex and for dimerization to occur. However, the results do not determine whether disulphide bonds are required for correct folding of the monomeric IFN-γs that enable dimerization, or alternatively,
Fig. 3. Disulphide bonds are needed for the formation of VV IFN-γR dimers. (a) 35S-labelled IFN-γR was prepared as in Fig. 2(a), mixed with Laemmli buffer in the presence or absence of 5% β-mercaptoethanol (ME) and analysed by SDS–PAGE and fluorography. Samples from insect cells infected with wild-type baculovirus (AcNPV) were analysed in parallel. (b) 35S-labelled IFN-γR was mixed with Laemmli buffer without reducing agent containing the indicated concentrations of NaCl or β-mercaptoethanol (ME), or at different pH. All samples were then analysed by SDS–PAGE and fluorography. The positions of monomeric or dimeric IFN-γRs are indicated. Molecular size markers are shown in kDa.

whether direct intermolecular C–C bonds mediate the formation of dimers.

The existence of the IFN-γR encoded by different orthopoxviruses as a dimer in the absence of ligand is likely to aid the ability of the virus protein to act as an inhibitor of IFN-γ. Support for this hypothesis comes from another soluble cytokine-binding protein encoded by poxviruses: the secreted TNF-binding protein from myxoma virus (M-T2) is present in solution as a monomer and a dimer, but the dimeric protein is a more potent inhibitor of TNF (Schreiber et al., 1996). In contrast to these examples, data presented here and elsewhere (Alcamí et al., 2000) indicate that the VV IFN-α/βR and the IL-1βR are monomers.

The dimerization of orthopoxvirus IFN-γRs is interesting in comparison to the cellular IFN-γR that dimerizes only after binding the dimeric ligand. Dimerization in the latter case is needed to trigger signal transduction. The crystal structure of the extracellular domain of the IFN-γR complexed with IFN-γ indicates that even after ligand binding the two IFN-γR chains remain separated by 27 Å (Walter et al., 1995). Yet although the cellular and poxvirus IFN-γRs have amino acid similarity and belong to the same superfamily, the orthopoxvirus protein exists as a dimer without ligand. The expression of dimeric IFN-γRs by the virus may increase the neutralization capacity of the soluble IFN-γRs. It is possible that this unique property of the VV protein is linked structurally with the ability of the protein to bind IFNs from a wide range of species, rather than being species-specific. Upon binding to its cellular receptor, IFN-γ undergoes a conformational change with the flexible AB loop changing to form a 3(10) α-helix (Walter et al., 1995), and it will be interesting to determine if a similar change accompanies binding to the virus IFN-γR. These issues require structural determination of the virus protein complexed with its ligand.

This work was supported by a programme grant from The Wellcome Trust. A.A. is a Wellcome Trust Senior Fellow and G.L.S is a Wellcome Trust Principal Research Fellow.

References


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Received 17 August 2001; Accepted 15 November 2001