JOURNAL OF VIROLOGY, Sept. 2005, p. 11205–11213 Vol. 79, No. 17
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.17.11205–11213.2005

Glycosylation, Disulfide Bond Formation, and the Presence of a WSXWS-Like Motif in the Orf Virus GIF Protein Are Critical for Maintaining the Integrity of Binding to Ovine Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-2

C. J. McInnes,* D. Deane, D. Haig, A. Percival, J. Thomson, and A. R. Wood

Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, Scotland, United Kingdom

Received 15 November 2004/Accepted 2 June 2005

Orf virus (ORFV), the type species of the family *Parapoxviridae*, encodes a protein (GIF) that binds and inhibits the ovine cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2). There is no obvious sequence homology between the ORFV protein and any known mammalian GM-CSF- or IL-2-binding proteins. We demonstrate here that many of the biochemical properties of mammalian GM-CSF receptors that are required for efficient binding of GM-CSF are also critical to the GIF protein for binding to ovine GM-CSF (ovGM-CSF). Site-directed mutagenesis of the GIF protein demonstrated, first, the importance of disulfide bonds, and second, that a sequence motif (WDPWV), related to the WSXWS motif of the type 1 cytokine receptor superfamily, was necessary for biological activity. Finally, glycosylation of the GIF protein was also critical for binding to GM-CSF.

Orf virus (ORFV) is a poxvirus that causes a debilitating skin disease in sheep, goats, and humans. It is found ubiquitously wherever sheep and goats are farmed, most frequently causing scabby lesions around the mouth and nares of suckling lambs and the teats of nursing ewes. Orf virus infection is rarely fatal in its own right, although secondary opportunistic infections can cause complications. It does, however, present a serious welfare problem to the animals and can lead to substantial economic loss for the farmer (18).

In common with other poxviruses, ORFV encodes several proteins that have the potential to interact with and/or subvert the host immune response to infection. The study of these viral proteins should provide insight into the ways in which mammalian hosts combat viral infections. In ORFV, genes for homologues of the mammalian proteins vascular endothelial growth factor (24) and interleukin-10 (IL-10) have been found (16), together with a double-stranded RNA-binding protein that inhibits the antiviral effects of interferon (17, 29), a chemokine-binding protein (33), and granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibitory factor (GIF), a protein which has been shown to bind and inhibit the ovine cytokines GM-CSF and IL-2 (10). This protein was the first cytokine-binding protein to be described in ORFV and suggests a role for these two cytokines in the host response to the virus infection. Other cytokine-binding proteins of poxvirus origin had been reported previously (1–3, 26). They generally fall into two categories: those that have obvious sequence homology to cellular cytokine receptors/binding proteins and those that do not. Sequence analysis suggested that the GIF protein does not resemble any known mammalian GM-CSF-binding or IL-2-binding proteins, and indeed, there are no reports of any other protein capable of binding both GM-CSF and IL-2. These cytokines share little homology at the primary amino acid level but do have a common structural topology characterized by four alpha helices. Their cellular receptors are also related, being members of the cytokine receptor superfamily, the cytokine-binding regions of which contain sequence, distantly related to the fibronectin type III domain, which forms a generic antiparallel β-sandwich structure analogous to the immunoglobulin constant domain (5). Disulfide bonds and a sequence motif known as the "WSXWS" box are crucial for maintaining the structural integrity of these receptors. Recently, it has been suggested that the GIF protein is a member of the poxvirus type II chemokine-binding protein (CBP) family (33), although no chemokine-binding activity has been shown for GIF. The type II CBPs are a family of related proteins which bind C-C chemokines but lack any obvious homology to cellular chemokine receptors. Structural analysis of the type II CBP from cowpox virus suggests that these proteins also form globular proteins consisting of an antiparallel β-sandwich that is intimately involved in the interaction with their ligands (6). We have performed structure-function studies to try to elucidate the mechanism by which the GIF protein is able to interact with GM-CSF and examined cytokine-binding activity in relation to the glycosylation state of GIF, the presence of individual cysteine residues, and also the sequence motif WDPWV found within the GIF molecule.

MATERIALS AND METHODS

Assay of GIF activity. The presence of biologically active GIF can be measured indirectly by its ability to interfere with the detection of ovine GM-CSF by specific enzyme-linked immunosorbent assay (ELISA) (15). Briefly, samples containing GIF and control samples are incubated with 4 ng/ml ovGM-CSF for

* Corresponding author. Mailing address: Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, Scotland, United Kingdom. Phone: 44 (0) 131 445 6130. Fax: 44 (0)131 445 6111. E-mail: mcinc@mri.sari.ac.uk.

TABLE 1. PCR primers used to produce site-directed mutants of GM-CSF and GIF

GIF construct	5′ primer	X-Ala mutation reverse primer (5′-3′)
Glu^{114} (Δ115-246)	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
Arg^{169} (Δ170-246)	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
Leu^{208} (Δ209-246)	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
His^{217} (Δ218-246)	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
Ser^{237} (Δ238-246)	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
Ser^{241} (Δ242-246)	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
$(Cys\text{-}Ala)^{10}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	TGGGCCGTGGCGAAGTCGCG
$(Cys\text{-}Ala)^{40}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	GCCAGCGCGGCGGCCGAGCT
$(Cys\text{-}Ala)^{71}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	AAGAAGCCGGCGTTGATCGC
$(Cys\text{-}Ala)^{136}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	GGCACCGTGGCGCCCACGCG
$(Cys\text{-}Ala)^{185}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	GCGCTCACGGCCCGGTGGTC
$(Cys\text{-}Ala)^{199}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	CGGGCGCTAGCGGCGTCCTG
$(Cys\text{-}Ala)^{215}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	GGATGGTTGGCGCCATGCGC
$(Cys\text{-}Ala)^{240}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	
$(Trp\text{-}Ala)^{97}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	CACCCAAGGGTCCGCGGAGTCCATGTACAC
$(Asp\text{-}Ala)^{98}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	GTCGATCACCCAAGGGGCCCAGGAGTCCAT
$(Trp\text{-}Ala)^{100}$	GGAAAGCTTGCGCCGGCTCTAGGAAAGAT	GGGTCGTCGATCACCGCAGGGTCCCAGGAG
GM-CSF construct		
$(Leu\text{-}Ala)^{23}$	GGGAAGCTTCCGCCATGTGGCTGCAGAACC	CGTTCAGAAGGCTCGCGGCCTCCTTGATGG
$(Ser\text{-}Ala)^{24}$	GGGAAGCTTCCGCCATGTGGCTGCAGAACC	CGTTCAGAAGGGCCAGGGCCTCCTTGATGG
$(Leu\text{-}Ala)^{23}$ $(Ser\text{-}Ala)^{24}$	GGGAAGCTTCCGCCATGTGGCTGCAGAACC	CGTTCAGAAGGGCCGCGGCCTCCTTGATGG

1 h at 37°C and then assayed by specific ELISA. The presence of biologically active GIF is indicated by a reduction in optical density for the known amount of ovine GM-CSF. When no biologically active GIF is present, there is no interference with GM-CSF detection. This correlates well with GIF being able to reduce both IL-2 and GM-CSF activities in T-cell proliferation assays and soft-agar clonogenic assays, respectively, and also in specific cytokine binding, as assayed by ligand blots (10). Here, cell-free supernatants and cell lysates from GIF cDNA-transfected and virus-infected cells were assayed for GM-CSF-binding activity as described previously (10). Binding of GIF to ovine IL-2 was also measured by ELISA. Briefly, IL-2 was isolated from CHO cell supernatants by Mono-Q anion-exchange chromatography, followed by gel filtration on a Sephacryl-200 HR column (Amersham Biosciences, Chalfont St. Giles, United Kingdom). ELISA plates were coated overnight with 1 μg/ml of IL-2 in 0.1 M $NaHCO_3$, pH 9.5, before being blocked with 4% nonfat milk powder in phosphate-buffered saline (PBS). Cell supernatants and cell lysates from GIF cDNA-transfected and virus-infected cells were assayed for IL-2-binding activity. Bound GIF was detected with 2 μg/ml of an affinity-purified immunoglobulin G (IgG) fraction from a rabbit anti-GIF serum, followed by a 1:1,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (DAKO, Ely, United Kingdom) in wash buffer (PBS containing 0.02% Tween 20 [Sigma]). For color development, TMB Peroxidase Substrate (SureBlue; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added; the reaction was stopped after 20 to 30 min by the addition of 0.1 N HCl and read at an optical density of 450 nm. The specificity of the interaction between GIF and IL-2 was verified by preincubating the IL-2-coated ELISA plate with an anti-ovine IL-2 antibody (1B3) before incubating it with GIF.

Production of GIF and GM-CSF mutants. A series of six COOH terminus deletions were prepared using PCR. Primers were used to introduce a stop codon into the GIF sequence after Glu^{114}, Arg^{169}, Leu^{208}, His^{217}, Ser^{237}, and Ser^{241} (numbering with respect to the mature secreted protein). The same 5′ primer was used in each instance. The primers used for each PCR are shown in Table 1. Each of the PCR products was ligated into the pEE14 expression vector (Celltech, Slough, United Kingdom) (8) and verified by sequencing prior to transfection into COS-7 or CHO cells using the Superfect (pfx7) transfection reagent (QIAGEN, Crawley, United Kingdom) following the manufacturer's recommended procedures. The transfected cells were maintained in glutamine-free Glasgow's modified Eagle's medium (Gibco BRL, Paisley, United Kingdom) with or without 7.5% heat-inactivated dialyzed fetal bovine serum (PAA Laboratories, Kingston upon Thames, United Kingdom) and methionine-sulfoxamine as appropriate (MSX; Sigma, Poole, United Kingdom).

Cys-Ala substitutions were introduced into the GIF protein using the site-directed mutagenesis PCR technique reported by Ho et al. (21). Trp^{97}-Ala^{97}, Asp^{98}-Ala^{98}, and Trp^{100}-Ala^{100} substitutions were also obtained in a similar way. Briefly, primers containing the Cys-, Trp-, or Asp-to-Ala substitutions were used in a primary PCR to produce two overlapping PCR fragments containing the sequence from the start of the protein to the region containing the residue to be substituted and from that region to the end of the protein. These PCR products were then used as targets for a secondary reaction using primers corresponding to the beginning and end of the complete GIF protein. The primers used for each of the PCRs are shown in Table 1. The only exception to this was the Cys^{240}-Ala^{240} substitution. This Cys residue is situated 6 amino acids from the end of the mature protein, and therefore, the Cys-Ala substitution was incorporated directly into the 3′-end primer and used in a conventional PCR. As with the COOH terminus deletion mutants, the PCR products were cloned into pEE14 and their sequences were verified to check that the appropriate substitution had been incorporated prior to expression of the mutated protein in COS-7 or CHO cells. GIF activity was assayed as described above in both cell supernatants and cell lysates. Cell lysates were obtained from washed cell pellets subjected to disruption in 1% NP-40–150 mM NaCl–50 mM Tris-HCl, pH 8.0 (1 ml/10^7 cells) for 15 min, followed by centrifugation at 12,000 × *g* for 10 min.

Leu^{23}-Ala^{23} and Ser^{24}-Ala^{24} substitutions were introduced into the ovine GM-CSF molecule as described above (numbering with respect to the mature protein). Production of the mutated ovine GM-CSF was monitored and quantified using the GM-CSF-specific ELISA (15).

Western blot analysis. In order to verify protein expression from the various GIF constructs, the cell supernatants and/or cell lysates from the COS-7/CHO cells were electrophoresed in a 12% denaturing SDS-polyacrylamide gel and transferred to BA 83 nitrocellulose membranes (Schleicher and Schuell, Anderman, Kingston upon Thames, United Kingdom) for 3 h at 2 mA/cm^2 of gel. The membranes were washed in PBS containing 4% nonfat milk powder (Marvel; Chivers, Dublin, Ireland) for 1 h at room temperature before being probed with a rabbit anti-GIF IgG fraction (1 μg/ml) in blot wash buffer (PBS containing 0.35 M NaCl and 0.5% [vol/vol] Tween 80 [Sigma, Poole, United Kingdom]). Binding of antibody to immobilized proteins was visualized by a further 1-h incubation with a 1:1,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (DAKO, Ely, United Kingdom) in wash buffer, followed by treatment with the enhanced-chemiluminescence reagent (Amersham Biosciences, Chalfont St. Giles, United Kingdom) according to the manufacturer's instructions and exposure to Hyperfilm ECL for 30 s to 5 min before development.

Ligand blotting and GM-CSF receptor-binding assay. GIF and ovine IL-2 were purified from CHO cell supernatants by Mono-Q anion-exchange chromatography, followed by gel filtration on a Sephacryl-200 HR column (Amersham Biosciences, Chalfont St. Giles, United Kingdom). Ovine GM-CSF was purified by affinity chromatography with purified IgG from monoclonal antibody (MAb) 3C2 (specific for ovine GM-CSF) (15) bound to Sepharose. For GIF-cytokine ligand blots and for GM-CSF receptor-binding studies, purified proteins were

Table 1—*Continued*

X-Ala mutation forward primer	3′ primer (5′-3′)
	GGGAATTCACTCCTGGCTGAAGAG
	ATGAATTCACCGGCGGTTCGGCCG
	CAGAATTCAGAGTGGCGTCCGCGC
	GGGAATTCAATGGTTGCAGCCATG
	AGGAATTCAGGAGATCGGCGACGA
	GAGAATTCAGCTGCACTTCCTGGA
CGCGACTTCGCCACGGCCCA	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
AGCTCGGCCGCCGCGCTGGC	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
GCGATCAACGCCGGCTTCTT	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
CGCGTGGGCGCCACGGTGCC	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
ACCACCGGGCCGTGAGCGC	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
CAGGACGCCGCTAGCGCCCG	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
GGGCATGGCGCCAACCATCC	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
	AAGAATTCAGCGCGCCGTCTGCATGCTGGCCTTCCTGGA
GTGTACATGGACTCCGCGGACCCTTGGGTG	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
ATGGACTCCTGGGCCCCTTGGGTGATCGAC	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
CTCCTGGGACCCTGCGGTGATCGACGACCC	TAAGAATTCAGCGCGCCGTCTGCATGCTGC
CCATCAAGGAGGCCGCGAGCCTTCTGAACG	GGGGAATTCTCACTTCTGGACTGGTTCCCA
CCATCAAGGAGGCCCTGGCCCTTCTGAACG	GGGGAATTCTCACTTCTGGACTGGTTCCCA
CCATCAAGGAGGCCGCGGCCCTTCTGAACG	GGGGAATTCTCACTTCTGGACTGGTTCCCA

radioiodinated by the chloramine-T method (25) and further purified on Sephacryl 200 HR columns in PBS with 0.15% (vol/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) buffer, pH 7.2.

GIF-cytokine binding was detected by ligand blotting as described previously (10). Briefly, 250 to 400 ng of each cytokine was separated by 15% denaturing SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked in blocking buffer, washed in PBS containing 0.05% Tween 20 (wash buffer), and then incubated with ^{125}I-GIF (15 to 25 pM) for 2 h at room temperature. After being washed three times with wash buffer, bound GIF was detected by autoradiography using Hyperfilm MP X-ray film (Amersham Biosciences).

Neutrophils were isolated from sheep peripheral blood, and binding of ^{125}I-GM-CSF to its native receptor present on the neutrophils was measured essentially by the method of Dower et al. (14). The cells were washed twice in binding buffer comprising RPMI 1640 (Gibco-BRL, Paisley, United Kingdom) containing 1% fetal calf serum and 20 mM HEPES, pH 7.5. They were then added at 2×10^6 in 50 μl to 250 μl of binding buffer containing ^{125}I-labeled GM-CSF (0.1 to 1 nM) and incubated for 2 h at 4°C. As controls, ^{125}I-labeled cytokines were also preincubated with an anti-GM-CSF MAb, 8D8, at 4°C for 60 min prior to being added to the neutrophils. Cells were isolated from the reaction mixture by centrifugation of the cell suspension through 400 μl of a phthalate-oil mixture comprising a 1.5:1 mixture of dibutyl phthalate-bis(2ethylhexyl)phthalate (Sigma, Poole, United Kingdom) at 13,000 × *g* for 1 min. The supernatant was carefully removed, the tip of the centrifuge tube containing the cell pellet was removed with a surgical blade, and the cell-bound radioactivity was measured in a γ counter.

Blocking or removal of Asn-linked carbohydrate from the GIF protein. Fetal lamb muscle (FLM) cells were infected with ORFV at a multiplicity of infection of 0.5 in the presence or absence of 20 μg/ml tunicamycin. Once >50% of the cells exhibited signs of infection, the cell supernatants were collected, and the cells themselves were gently washed twice with PBS at 800 × *g*. The cell pellet was then frozen overnight, and cell lysates were prepared as before.

Aliquots of purified recombinant GIF (10 to 20 μg) in 200 μl of 200 mM sodium phosphate buffer, pH 7.2, containing 10 mM EDTA, 1% (vol/vol) 2-mercaptoethanol, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) Triton X-100 (Sigma) were boiled for 5 min. The mixtures were allowed to cool before the addition of 1 U glycopeptidase F (PNGase F; Sigma) and incubation for 24 h at 37°C. Samples (10 μl) were analyzed for a shift in mobility either by SDS-polyacrylamide gel electrophoresis (PAGE), followed by silver staining, or by Western blotting.

RESULTS

Residues in the ovine GM-CSF molecule that interact with its native receptor and the ORFV GIF molecule. The two residues Leu^{23} and Ser^{24} of the mature ovine GM-CSF molecule that we had predicted previously to be involved in the interaction of the cytokine and its cellular receptor (28) were individually replaced with an Ala residue, and the mutated forms of GM-CSF were expressed in CHO cells. The proteins were purified, quantified by specific ELISA, labeled with ^{125}I, and incubated with a neutrophil fraction isolated from ovine peripheral blood lymphocytes. The labeled GM-CSF bound to the neutrophils was quantified by γ counting, and the results are shown in Fig. 1a and b. No significant binding of GM-CSF Leu^{23}-Ala^{23} to the neutrophils was detected, whereas binding of GM-CSF Ser^{24}-Ala^{24} was comparable to that of the native GM-CSF.

We investigated whether the GIF molecule could bind to these mutants by preincubating them with GIF prior to performing the GM-CSF-specific ELISA. The results are presented in Fig. 1c. GM-CSF Leu^{23}-Ala^{23} did not bind to the GIF molecule, whereas binding to GM-CSF Ser^{24}-Ala^{24} was indistinguishable from that to the native GM-CSF.

Residues in the ORFV GIF molecule that influence binding to GM-CSF. In order to examine regions of the GIF molecule that were critical to its binding activity, a number of COOH terminus, Cys-Ala, and other site-directed mutants of the GIF protein were expressed and the ability of each to bind GM-CSF was assayed using the ovGM-CSF-specific ELISA (15). Cell supernatants and cell lysates were collected from each batch of transformed cells and concentrated at least threefold. The supernatants were preincubated with 4 ng recombinant ovine GM-CSF for 1 h prior to performing the GM-CSF-specific ELISA. The COOH terminus deletion mutants Ser^{237} (GIFΔ238-246) and Ser^{241}(GIFΔ242-246) were designed so

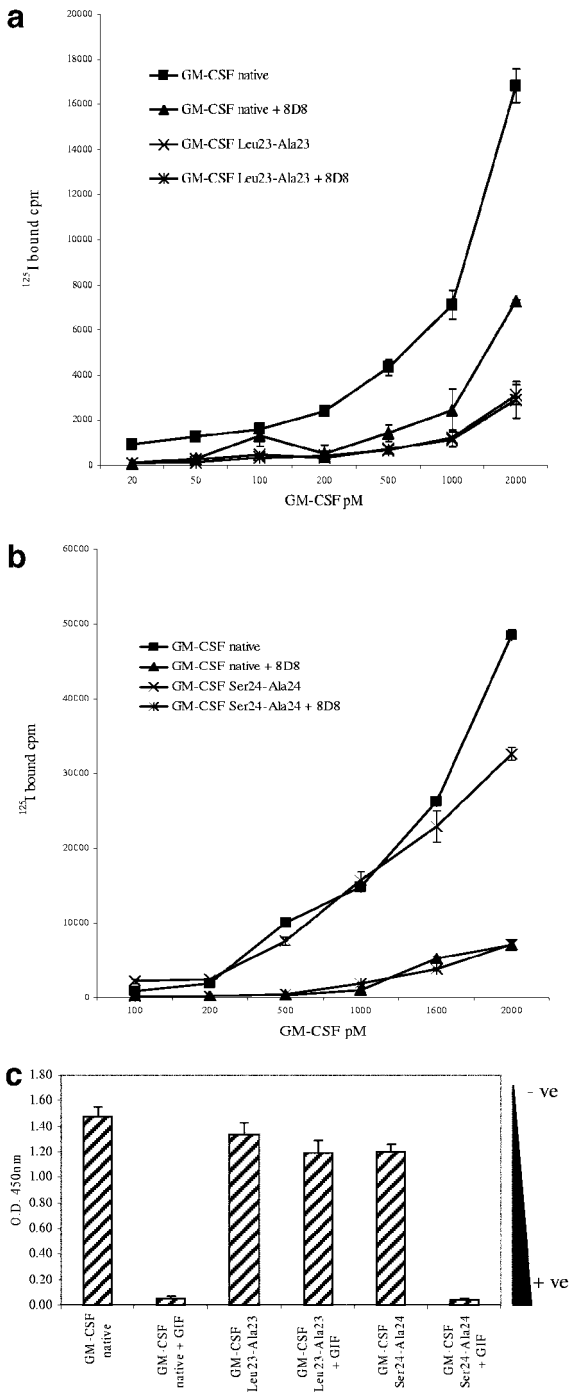

FIG. 1. Site-directed mutants of ovine GM-CSF were tested for the ability to bind to ovine neutrophils and the ORFV GIF protein. (a and b) ^{125}I-labeled native ovine GM-CSF and the two Ala substitution mutants were incubated with ovine neutrophils. Binding was assessed by measuring the cell-bound radioactivity in a γ counter. Preincubation of the samples with an anti-GM-CSF MAb (8D8), which inhibits binding of GMCSF to neutrophils, was included as a control. (c) GM-CSF-specific ELISA demonstrating the ability of the GIF molecule to bind both native ovine GM-CSF and GM-CSF Ser^{24}-Ala^{24}. The GIF protein did not bind GM-CSF Leu^{23}-Ala^{23}. The error bars indicate standard errors of the mean. The +ve/−ve scale indicates binding of GIF to cytokine.

that Ser^{241} included the last of the eight Cys residues found in the mature GIF protein (Cys^{240}) whereas Ser^{237} stopped before this Cys residue. Ser^{241} was the only COOH terminus deletion mutant to demonstrate any capacity for binding GM-CSF (results not shown), and thus, indicated the importance of Cys^{240} to the biological activity of the GIF protein. We therefore replaced each of the Cys residues found in the mature GIF protein individually in turn with an Ala residue to determine which others, if any, were required for the biological activity. The results of the GM-CSF-specific ELISA with each of the Cys-Ala substitution mutants are presented in Fig. 2a. Transfections with each of the GIF constructs were performed at least in triplicate to verify the presence or absence of GM-CSF-binding activity. Six of the eight Cys residues, Cys^{10}, Cys^{40}, Cys^{136}, Cys^{185}, Cys^{199}, and Cys^{240}, were found to be critical for ligand-binding activity. GIF Cys^{215}-Ala^{215} appeared to have the same capacity to bind GM-CSF as the wild-type protein, and GIF Cys^{71}-Ala^{71} appeared to have a slightly reduced capacity for binding. To verify whether the same Cys-Ala substitutions affected binding of GIF to IL-2, cell supernatants and lysates from GIF-transfected cells were incubated with ovine IL-2 immobilized on an ELISA plate. After being washed, GIF bound to IL-2 was detected with an antibody specific for the GIF molecule (Fig. 2b). Again, only Cys^{71} and Cys^{215} did not appear critical for IL-2-binding activity, while replacement of the other Cys residues with Ala residues resulted in an inactive protein. In order to demonstrate that recombinant protein was being produced for each of the GIF Cys-Ala mutants, the cell supernatants and the cell lysates from each of the transfections were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-GIF antiserum. The resulting Western blots are shown in Fig. 2c. GIF protein was detected in all of the Cys-Ala substitution mutants. It appeared, however, that some of the substitutions resulted in the GIF protein being retained within the cell. The Cys-Ala-mutated GIF proteins also appeared to have been produced in various amounts and to vary slightly in molecular weight. The variation in the amount of protein may be an experimental artifact due to differing transfection efficiencies, but it could, together with the differences observed in the molecular weights, indicate that disruption of the tertiary structure due to the loss of a disulfide bond might affect the efficiency of protein production and the post- or cotranslational modification of the protein.

Close examination of the predicted amino acid sequence of the mature GIF protein revealed the presence of the sequence WDPWV. This resembles a sequence, WSXWS, that is conserved across a number of cytokine receptors that belong to the cytokine receptor superfamily and that is involved in maintaining the surface expression of membrane receptors and the secretion of soluble receptors. We replaced each of the Trp residues (Trp^{97} and Trp^{100}) and the Asp^{98} residue in the GIF sequence individually in turn with an Ala residue and assayed the recombinant protein for its ability to bind ovGM-CSF. The results are presented in Fig. 3a. ORFV GIF Trp^{97}-Ala^{97} was able to bind ovGM-CSF, but neither the GIF Trp^{100}-Ala^{100} nor the GIF Asp^{98}-Ala^{98} mutant bound ovGM-CSF in solution. Since it appears that mutations within the WSXWS motif can result in proteins being trapped within the cell (20), we checked whether the mutated GIFs could be detected in the

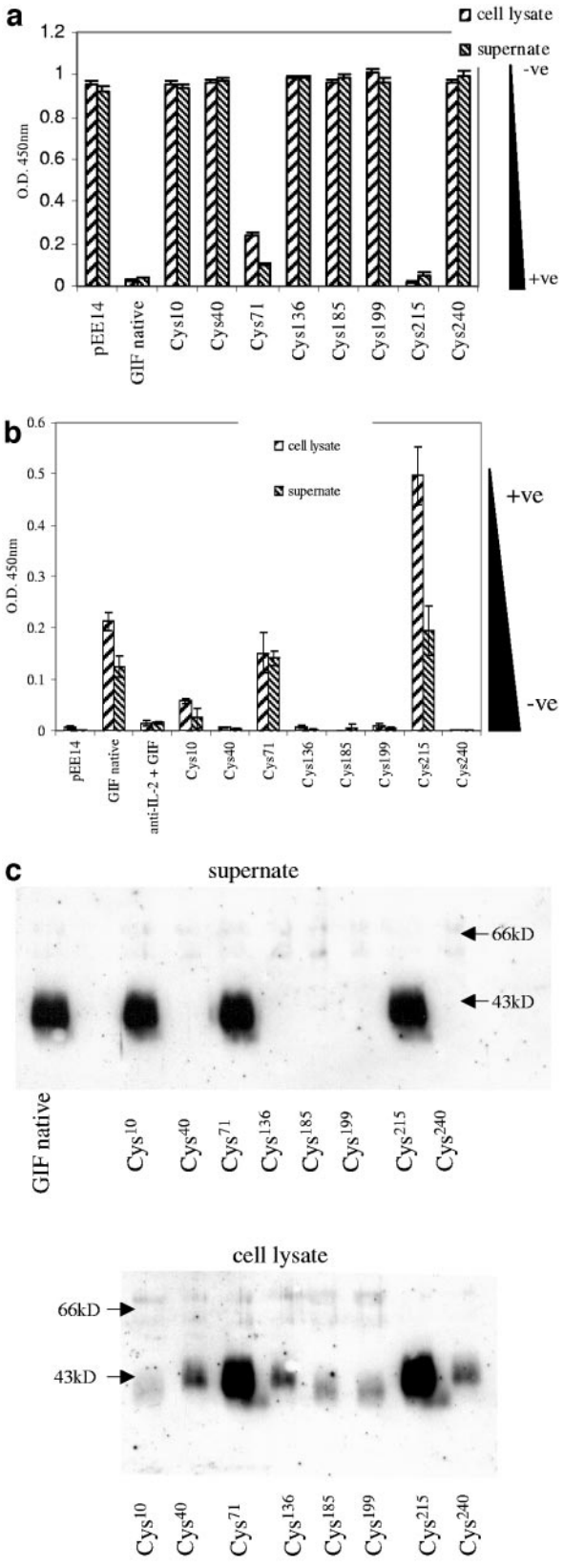

FIG. 2. Cys-Ala substitution mutants of the GIF molecule were assessed, using the GM-CSF-specific ELISA, for the ability to bind native GM-CSF. Cell supernatants and cell lysates were collected from CHO cells transfected with various site-directed mutants of the GIF gene expressed from the mammalian expression vector pEE14. The pEE14 plasmid, containing no GIF insert, was included as a control. (a) Cys-Ala substitution mutants were tested for the ability to block detection of GM-CSF. Only GIF Cys^{71}-Ala^{71} and GIF Cys^{215}-Ala^{215} were able to do so. (b) Cys-Ala substitution mutants were tested for the ability to bind ovine IL-2 immobilized on an ELISA plate. GIF bound to IL-2 was detected with an anti-GIF antibody. Only GIF Cys^{71}-Ala^{71} and GIF Cys^{215}-Ala^{215} were able to bind to IL-2. The specificity of the interaction between GIF and IL-2 was verified by preincubating the IL-2-coated ELISA plate with an anti-ovine-IL-2 antibody (1B3) before incubating it with GIF. (c) Western blot of the cell supernatants and cell lysates from panel a. The error bars indicate standard errors of the mean. The +ve/−ve scale indicates binding of GIF to cytokine. O.D., optical density.

cell lysates. A Western blot of the cell lysates together with the cell supernatants is shown in Fig. 3b. All three mutated GIFs were detected both in the cell lysates and in the cell supernatants, although from superficial examination of the blot it appears that there is more GIF Asp^{98}-Ala^{98} and Trp^{100}-Ala^{100} within the cell than is being secreted from the cell. Therefore, we postulated that the lack of detectable binding activity shown by these mutants may have been due to the low concentrations of GIF protein found in the cell supernatants. To verify that GIF Trp^{100}-Ala^{100} was indeed incapable of binding GM-CSF, we purified it from the supernatant and used it as a probe in a ligand blot of ovGM-CSF and ovIL-2. The results are presented in Fig. 3c. Whereas native GIF and GIF Trp^{98}-Ala^{98} were capable of binding to both ovGM-CSF and ovIL-2 immobilized on nitrocellulose, purified GIF Trp^{100}-Ala^{100} was not.

Asn-linked carbohydrate on the GIF protein is required for biological activity. The GIF protein was produced in vitro by ORFV infection of FLM cells grown in the presence or absence of tunicamycin, an inhibitor of Asn glycosylation. A Western blot of the resulting protein, presented in Fig. 4a, shows that there is a shift due to Asn-linked carbohydrate of approximately 10 kDa. The results also indicate that the GIF protein produced in the cells treated with tunicamycin is not readily exported from the cell. Similarly, when purified recombinant GIF is treated with the glycopeptidase PNGase F, there is a reduction in apparent molecular mass from approximately 43 kDa to approximately 33 kDa, indicating that about 25% of the apparent molecular mass of the GIF protein is likely to be due to Asn-linked carbohydrate residues (Fig. 4b). In order to test whether the Asn-linked carbohydrate influences the binding activity of the GIF protein, cell supernatants and cell lysates from the ORFV-infected cells (with and without tunicamycin treatment) were tested for the ability to prevent GM-CSF detection in the specific ELISA (Fig. 4c). The GIF produced from cells treated with tunicamycin was unable to bind GM-CSF. Similarly, when incubated with recombinant ovine IL-2 immobilized on an ELISA plate, the nonglycosylated GIF did not bind (Fig. 4d).

Structural prediction for GIF. A structural prediction for the GIF molecule was produced using the mGenTHREADER Fold Recognition program (27). This program uses the outputs from PSI-BLAST and PSIPRED to look for structurally similar proteins in the Research Collaboratory for Structural Bioinformatics protein data bank. The only significant match

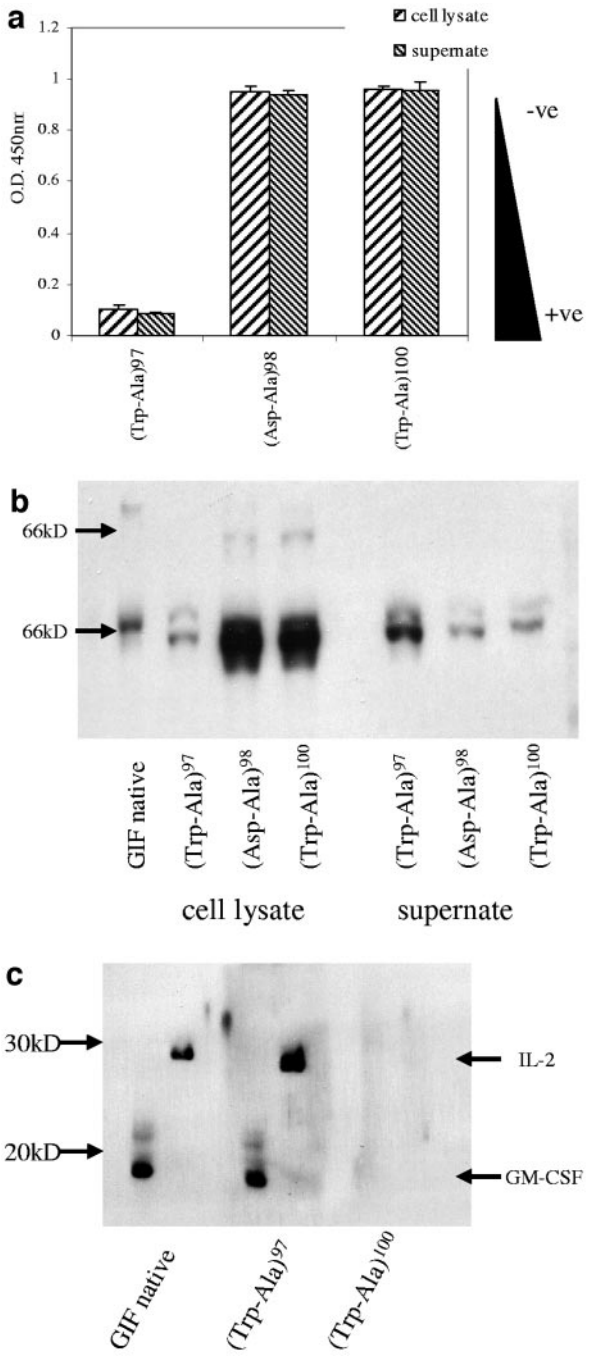

FIG. 3. GIF proteins containing mutations in the "WDPWV" motif were tested for the ability to bind GM-CSF. Cell supernatants and cell lysates were collected from CHO cells transfected with various site-directed mutants of the GIF gene expressed from the mammalian expression vector pEE14. (a) The Trp^{97}-Ala^{97}, Asp^{98}-Ala^{98}, and Trp^{100}-Ala^{100} substitution mutants were tested for the ability to block GM-CSF detection in the specific ELISA. (b) Western blot of the three substitution mutants from panel a. (c) Ovine GM-CSF and IL-2 were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were incubated with ^{125}I-labeled native GIF, as well as the ^{125}I-labeled Trp^{97}-Ala^{97} and Trp^{100}-Ala^{100} substitution mutants. The error bars indicate standard errors of the mean. The +ve/−ve scale indicates binding of GIF to cytokine. O.D., optical density.

found was with the *Cowpox virus* chemokine-binding protein (vCCI) (6). The prediction of secondary structure (PSIPRED) suggests that the GIF protein is composed mainly of β-strands that align with those found in the vCCI protein (Fig. 5). Although the GIF molecule, like the vCCI molecule, has eight Cys residues, only seven of these are positionally conserved. This fits well with the site-directed mutagenesis of the Cys residues, suggesting that the GIF protein has only three of the four disulfide linkages present in vCCI.

DISCUSSION

Sequence comparison of the GIF protein with known mammalian GM-CSF α or β_c, and IL-2 α, β,or γ_c receptor subunits provided no evidence that GIF was a viral orthologue of any of these proteins. Nevertheless, we sought to determine whether the interaction between GM-CSF and GIF was in any way analogous to the interaction between GM-CSF and its cellular receptor. The human receptor is comprised of two subunits, the low-affinity cytokine-specific α subunit and the β_c subunit (shared with the IL-3 and IL-5 receptors) that is required to form the high-affinity receptor. Both the α and β_c subunits are members of the type I cytokine receptor superfamily. Residues in the fourth α helix of the murine and human GM-CSF molecules are involved in interaction with the receptor α subunit, whereas residues in the first α helix have been shown to be important for formation of the high-affinity receptor (7, 19, 22, 23). Nothing was known about the interaction between ovine GM-CSF and its native receptor. However, we previously reported that sheep cells did not respond to human GM-CSF and that this was most likely due to an inability of the human GM-CSF to bind to the ovine receptor. Few differences were found between the sequences of ovine and human GM-CSF in the region predicted to form the fourth α helix, and therefore, we speculated that the lack of activity of human GM-CSF on ovine cells could be due to the two residues in the first α helix that differed between the ovine and human GM-CSFs (28). Using this as a starting point, in this study, we mutated the corresponding amino acids in the ovine GM-CSF molecule and found that one mutation (Leu^{23}-Ala^{23}) resulted in a protein that was no longer able to bind to the GM-CSF receptor on ovine neutrophils. The GIF molecule was also unable to bind this mutant, indicating that the same region of the GM-CSF molecule was likely to be involved in binding to both its cellular receptor and the GIF molecule.

Both the GM-CSF and IL-2 receptors are members of the type 1 cytokine receptor superfamily. The members of this family of receptors share little primary sequence homology but have a common structural cytokine-binding domain characterized by four spatially conserved cysteine residues and a set of spaced aromatic residues known as the WSXWS box. The cytokine-binding domain consists of approximately 200 amino acids, representing a duplicated ~100-amino-acid structure containing seven β-strands, and is predicted to form an antiparallel β-sandwich, with the WSXWS box being critical for maintaining the tertiary structure of the domain (5). The Cys residues in the GIF molecule do not have the same spatial arrangement as those in the cytokine receptor superfamily, although the site-directed mutagenesis experiments proved that at least six were absolutely required to maintain biological

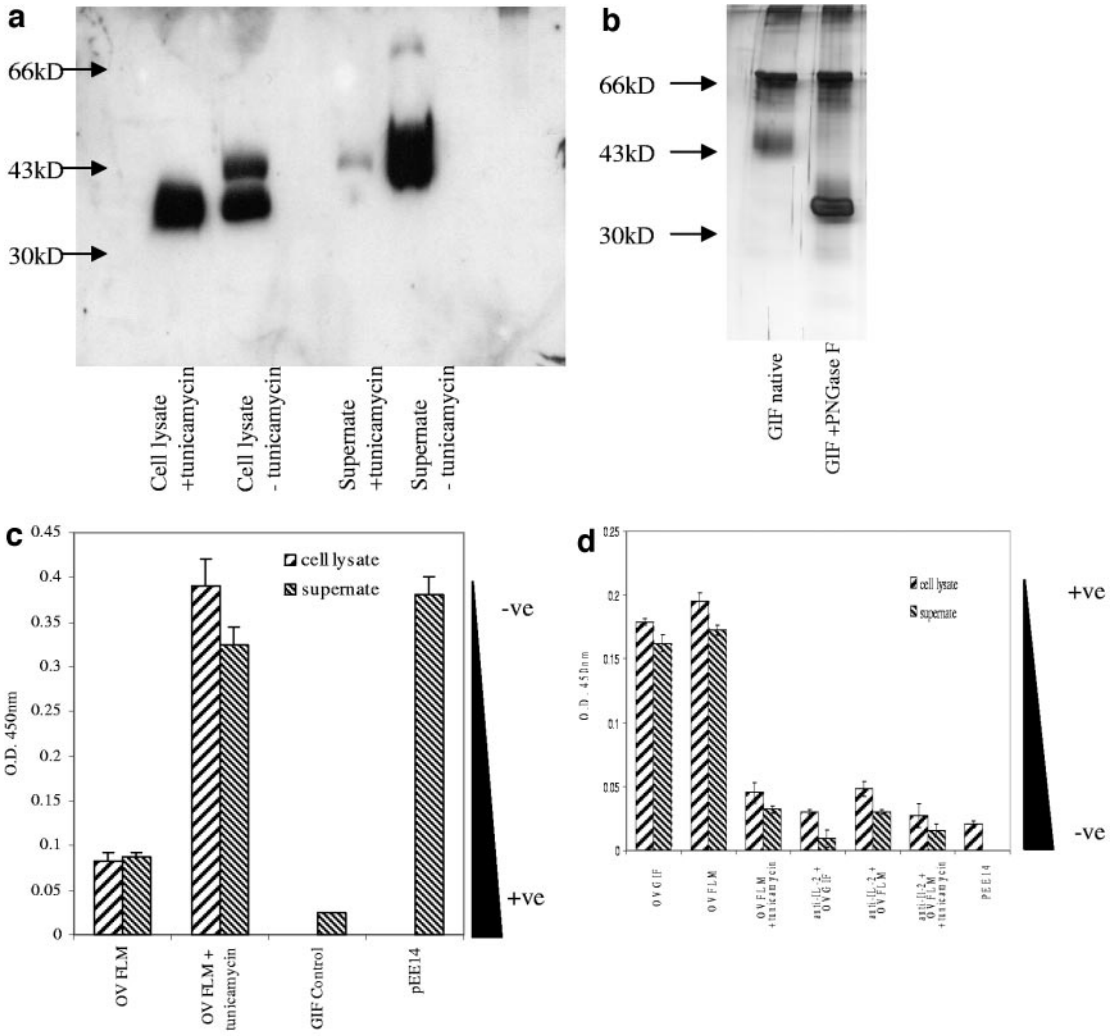

FIG. 4. The effect on GM-CSF binding of Asn-linked glycosylation of the GIF protein was tested in the GM-CSF-specific ELISA. (a) GIF protein was produced in vitro by infecting FLM cells with ORFV at a multiplicity of infection of 0.5. In order to block the addition of Asn-linked carbohydrate, the cells were treated with 20 μg/ml tunicamycin. A Western blot of the cell supernatants and cell lysates from treated and untreated cells is shown. (b) Recombinant GIF protein was produced in vitro and treated with PNGase F to remove Asn-linked carbohydrate. A silver-stained SDS polyacrylamide gel of native and treated GIF is shown. (c) The ability to block detection of GM-CSF by ELISA of the nonglycosylated form of the GIF protein from ORFV-infected FLM cells treated with tunicamycin was tested. Native recombinant GIF and cell supernatants and cell lysates from untreated cells were included as controls. (d) Binding of the nonglycosylated form of GIF was tested for its ability to bind ovine IL-2 immobilized on an ELISA plate. Bound GIF was detected using an anti-GIF antiserum. The error bars indicate standard errors of the mean. The +ve/−ve scale indicates binding of GIF to cytokine. O.D., optical density.

activity. These six Cys residues are also conserved in the GIF protein from *Bovine papular stomatitis virus* (BPSV), another of the *Parapoxviridae*, whereas the remaining two Cys residues that are not important for biological activity are not conserved (11). We presume that the Cys residues that are important for biological activity form three intramolecular disulfide bonds. Western blot results suggested that disruption of the correct disulfide bond formation was likely to lead to the protein being trapped within the cell, possibly also interfering with co- and posttranslational modification of the protein but suggesting that correct folding of the GIF molecule is required before it is secreted from the cell.

The WSXWS box is found in the majority of human and murine type 1 cytokine receptors that have been studied, but variants of the motif exist with both individual Trp residues and Ser residues subject to change (13). Although early evidence indicated that the WSXWS box may be directly involved in the interaction between the ligand and the receptor, more recent studies have suggested that its role is in stabilizing the conformation of the cytokine-binding domain. Although mutations within the motif apparently resulted in IL-2, IL-6, GM-CSF, and erythropoietin (EPO) receptors that were unable to bind their ligands, it is likely that this was a result of structural changes which either did not allow efficient transport of the receptors through the secretory pathway or occurred in receptors which were unable to form complexes with coreceptors (32, 34). Saturation mutagenesis of the EPO receptor has subsequently shown that mutations in the WSXWS box affected

FIG. 5. Alignment of the GIF sequence with that of the vCCI produced by mGenTHREADER (27). Gaps (-) were introduced into both sequences to produce the optimal alignment. Secondary-structure predictions for each sequence are shown above and below the respective sequences. E, β-sheet; H, helix; C, coil.

the efficiency with which the protein could exit the endoplasmic reticulum and hence the extent to which the protein was expressed at the cell surface (20). One of the mutations actually resulted in an increase in surface expression of the EPO receptor. Therefore, mutations within the WSXWS box can result in at least three receptor phenotypes: (i) receptors that are trapped within the endoplasmic reticulum, (ii) receptors that are expressed normally but that are deficient in ligand binding, and (iii) receptors that are expressed normally and bind their ligand with slightly altered efficiency (20). The GIF molecule possesses the motif WDPWV. Recent sequencing of the BPSV genome (11) revealed that the corresponding region of the BPSV GIF molecule contains the sequence WSPWT, which is obviously a closer match to the consensus than is found in the ORFV molecule and also indicates that this motif does indeed warrant further investigation. While mutation of the first Trp residue had no affect on the ability of GIF to bind GM-CSF, mutation of both the Asp residue and the second Trp residue resulted in GIF proteins which were unable to bind GM-CSF or IL-2. It appears that mutating these residues also resulted in a large proportion of the GIF molecule being retained within the cell.

The apparent molecular masses of many of the type 1 cytokine receptor subunits are far greater than the theoretical masses calculated from the primary amino acid sequences alone. In the majority of cases, this is due to Asn-linked glycosylation (9, 12). For example, between 11 kDa and 35 kDa of the apparent mass of the IL-2γ subunit appears to be due to carbohydrate, while Asn-linked carbohydrate has been shown to be responsible for 7% and 45% of the total molecular mass of the GM-CSF β and α receptor subunits, respectively. Asn-linked glycosylation is known to affect protein folding and trafficking (4), although it appears that the nonglycosylated forms of the GM-CSF receptor subunits can still be transported to the plasma membrane. Despite this, the nonglycosylated form of the GM-CSFα receptor cannot bind GM-CSF and the nonglycosylated form of the β subunit does not associate with native α subunits to form the high-affinity receptor (31). The GIF molecule has four potential Asn-linked glycosylation sites. Expression of both the recombinant and native GIF molecules results in a protein of approximately 43 kDa, 15 kDa larger than would be predicted from the primary amino acid sequence alone. Blocking Asn-linked glycosylation by the use of tunicamycin or using glycopeptidase F to remove Asn-linked carbohydrate reduces the size of the GIF protein to approximately 33 kDa and results in a protein which is both inactive and retained within the cell.

It was not possible to target residues within the GIF protein which might be involved in a specific interaction between it and either GM-CSF or IL-2, mainly because there was very little primary amino acid sequence similarity between GIF and the type 1 cytokine receptors. As a result, we concentrated on known features of the GIF molecule that might have an influence on its binding activity. As it turned out, disulfide bond formation, glycosylation, and the sequence motif WDPWV all seemed to be important for biological activity, but rather than disrupting a specific interaction, our site-directed mutations appear to have affected the proper folding and trafficking of the GIF protein. Similar effects were seen when glycosylation, disulfide bonds, and the WSXWS motif were disrupted in the type 1 cytokine superfamily of receptors (20), and this indicates a closer relationship between this family of receptors and the GIF protein than could have been predicted from a simple comparison of their sequences. It is hoped that a subsequent X-ray crystallographic study of the GIF protein on its own and in combination with either GM-CSF or IL-2 will inform us of the specific interactions between GIF and its ligands.

Attempts made to model the ORFV GIF on the known crystallographic structures of cytokine receptors failed, as there was not enough homology at the primary amino acid sequence level. Instead, a search of the sequence databases suggested that the protein most similar to GIF is the C-C chemokine-binding protein from ORFV (33). Furthermore, the tertiary-structure prediction tool mGenTHREADER (27) suggested that the GIF protein was related to the Type II (p35) chemokine-binding proteins of the ortho- and leporipoxviruses, with the GIF sequence being able to be threaded onto the known crystal structure of the representative member of this family (vCCI) from *Cowpoxvirus* (6 and data not shown). However, the reliability of this structure prediction must be called into question, as an exhaustive Smith-Waterman search of the databases with the GIF sequence failed to find a significant relationship between GIF and the poxvirus C-C chemokine-binding family of proteins. Instead, the relationship between the ORFV GIF and vCCI becomes apparent only after several iterations of BLAST database searching. The apparent relationship is due in the most part to the fact that there is some sequence homology between the GIF molecule and the vaccinia virus (VACV) A41L-like family of proteins, which in turn have some sequence homology to the vCCI protein. The regions of greatest homology, however, are different. Despite this, the sequence homology with the ORFV C-C chemokine-binding protein seems to suggest that the relationship with the vCCI protein may be valid, but this is balanced by the fact that GIF and the VACV A41L protein do not appear to bind any of the known classes of chemokines. A41L also does not bind GM-CSF or IL-2, and in fact, no ligand has so far been identified for this protein (30). It may well be that the ORFV GIF

and the VACV A41L-like family of proteins represent a class of cytokine-binding proteins distantly related to the C-C chemokine-binding proteins but with a distinct binding profile. Pairwise comparison of the GIF protein with the A41L family of proteins revealed that the sequence DPW from the "WDPWV" motif, which we have shown to be important for the biological activity of GIF, is conserved in these proteins but not in the C-C chemokine-binding proteins, further reinforcing the suggestion that this family of proteins has evolved a separate biological function. Elucidation of the exact interaction between the GIF molecule and its ligands will require crystallization of the protein, which in turn may inform us of possible ligands for the A41L family of proteins. In conclusion, we believe that GIF functions as a soluble, secreted receptor for GM-CSF and IL-2 that has retained key ligand-binding properties of host type 1 cytokine receptors while adapting to bind two cytokines of particular importance to host immunity to infection. The exact roles of these cytokines in the host response to an ORFV infection are being investigated by use of a recombinant virus lacking the *GIF* gene.

ACKNOWLEDGMENT

This work is funded by the Scottish Executive Environment and Rural Affairs Department.

REFERENCES

1. **Alcami, A., and G. L. Smith.** 1995. Cytokine receptors encoded by poxviruses—a lesson in cytokine biology. Immunol. Today **16:**474–478.
2. **Alcami, A., J. A. Symons, A. Khanna, and G. L. Smith.** 1998. Poxviruses: capturing cytokines and chemokines. Semin. Virol. **8:**419–427.
3. **Alcami, A., J. A. Symons, and G. L. Smith.** 2000. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. J. Virol. **74:**11230–11239.
4. **Bastian, W., J. Zhu, B. Way, D. Lockwood, and J. Livingston.** 1993. Glycosylation of Asn397 or Asn418 is required for normal insulin receptor biosynthesis and processing. Diabetes **42:**966–974.
5. **Bazan, J. F.** 1990. Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA **87:**6934–6938.
6. **Carfi, A., C. A. Smith, P. J. Smolak, J. McGrew, and D. C. Wiley.** 1999. Structure of a soluble secreted chemokine inhibitor vCCI (p35) from cowpox virus. Proc. Natl. Acad. Sci. USA **96:**12379–12383.
7. **Clark-Lewis, I., A. F. Lopez, L. B. To, M. A. Vadas, J. W. Schrader, L. E. Hood, and S. B. Kent.** 1988. Structure-function studies of human granulocyte-macrophage colony-stimulating factor. Identification of residues required for activity. J. Immunol. **141:**881–889.
8. **Cockett, M. I., C. R. Bebbington, and G. T. Yarranton.** 1990. High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamine synthetase gene amplification. Biotechnology **8:**662–667.
9. **Cole, A. R., N. E. Hall, H. R. Treutlein, J. S. Eddes, G. E. Reid, R. L. Moritz, and R. J. Simpson.** 1999. Disulfide bond structure and N-glycosylation sites of the extracellular domain of the human interleukin-6 receptor. J. Biol. Chem. **274:**7207–7215.
10. **Deane, D., C. J. McInnes, A. Percival, A. Wood, J. Thomson, A. Lear, J. Gilray, S. Fleming, A. Mercer, and D. Haig.** 2000. Orf virus encodes a novel secreted protein inhibitor of granulocyte-macrophage colony-stimulating factor and interleukin-2. J. Virol. **74:**1313–1320.
11. **Delhon, G., E. R. Tulman, C. L. Afonso, Z. Lu, A. de la Concha-Bermejillo, H. D. Lehmkuhl, M. E. Piccone, G. F. Kutish, and D. L. Rock.** 2004. Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. J. Virol. **78:**168–177.
12. **Ding, D. X., J. C. Vera, M. L. Heaney, and D. W. Golde.** 1995. N-glycosylation of the human granulocyte-macrophage colony-stimulating factor receptor alpha subunit is essential for ligand binding and signal transduction. J. Biol. Chem. **270:**24580–24584.
13. **Doshi, P. D., and J. F. DiPersio.** 1994. Three conserved motifs in the extracellular domain of the human granulocyte-macrophage colony-stimulating factor receptor subunit are essential for ligand binding and surface expression. Blood **84:**2539–2553.
14. **Dower, S. K., S. R. Kronheim, C. J. March, P. J. Conlon, T. P. Hopp, S. Gillis, and D. L. Urdal.** 1985. Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. J. Exp. Med. **162:**501–515.
15. **Entrican, G., D. Deane, M. MacLean, L. Inglis, J. Thomson, C. McInnes, and D. M. Haig.** 1996. Development of a sandwich ELISA for ovine granulocyte/macrophage colony-stimulating factor. Vet. Immunol. Immunopathol. **50:**105–115.
16. **Fleming, S. B., C. A. McCaughan, A. E. Andrews, A. D. Nash, and A. A. Mercer.** 1997. A homolog of interleukin-10 is encoded by the poxvirus orf virus. J. Virol. **71:**4857–4861.
17. **Haig, D. M., C. J. McInnes, J. Thomson, A. Wood, K. Bunyan, and A. Mercer.** 1998. The orf virus OV20.0L gene product is involved in interferon resistance and inhibits an interferon-inducible, double-stranded RNA-dependent kinase. Immunology **93:**335–340.
18. **Haig, D. M., and A. A. Mercer.** 1998. Ovine diseases. Orf. Vet. Res. **29:**311–326.
19. **Hercus, T. R., B. Cambareri, M. Dottore, J. Woodcock, C. J. Bagley, M. A. Vadas, M. F. Shannon, and A. F. Lopez.** 1994. Identification of residues in the first and fourth helices of human granulocyte-macrophage colony-stimulating factor involved in biologic activity and in binding to the alpha- and beta-chains of its receptor. Blood **83:**3500–3508.
20. **Hilton, D. J., S. S. Watowich, L. Katz, and H. F. Lodish.** 1996. Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor. J. Biol. Chem. **271:**4699–4708.
21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
22. **Kaushansky, K., S. G. Shoemaker, S. Alfaro, and C. Brown.** 1989. Hematopoietic activity of granulocyte/macrophage colony-stimulating factor is dependent upon two distinct regions of the molecule: functional analysis based upon the activities of interspecies hybrid growth factors. Proc. Natl. Acad. Sci. USA **86:**1213–1217.
23. **Lopez, A. F., M. F. Shannon, T. Hercus, N. A. Nicola, B. Cambareri, M. Dottore, M. J. Layton, L. Eglinton, and M. A. Vadas.** 1992. Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. EMBO J. **11:**909–916.
24. **Lyttle, D. J., K. M. Fraser, S. B. Fleming, A. A. Mercer, and A. J. Robinson.** 1994. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J. Virol. **68:**84–92.
25. **McConahey, P. J., and F. J. Dixon.** 1980. Radioiodination of proteins by the use of the chloramine-T method. Methods Enzymol. **70:**210–213.
26. **McFadden, G., and P. M. Murphy.** 2000. Host-related immunomodulators encoded by poxviruses and herpesviruses. Curr. Opin. Microbiol. **3:**371–378.
27. **McGuffin, L. J., and D. T. Jones.** 2003. Improvement of the GenTHREADER method for genomic fold recognition. Bioinformatics **19:**874–881.
28. **McInnes, C. J., and D. M. Haig.** 1991. Cloning and expression of a cDNA encoding ovine granulocyte-macrophage colony-stimulating factor. Gene **105:**275–279.
29. **McInnes, C. J., A. R. Wood, and A. A. Mercer.** 1998. Orf virus encodes a homolog of the vaccinia virus interferon-resistance gene E3L. Virus Genes **17:**107–115.
30. **Ng, A., D. C. Tscharke, P. C. Reading, and G. L. Smith.** 2001. The vaccinia virus A41L protein is a soluble 30 kDa glycoprotein that affects virus virulence. J. Gen. Virol. **82:**2095–2105.
31. **Niu, L., M. L. Heaney, J. C. Vera, and D. W. Golde.** 2000. High-affinity binding to the GM-CSF receptor requires intact N-glycosylation sites in the extracellular domain of the beta subunit. Blood **95:**3357–3362.
32. **Ronco, L. V., S. L. Silverman, S. G. Wong, D. J. Slamon, L. S. Park, and J. C. Gasson.** 1994. Identification of conserved amino acids in the human granulocyte-macrophage colony-stimulating factor receptor alpha subunit critical for function. Evidence for formation of a heterodimeric receptor complex prior to ligand binding. J. Biol. Chem. **269:**277–283.
33. **Seet, B. T., C. A. McCaughan, T. M. Handel, A. Mercer, C. Brunetti, G. McFadden, and S. B. Fleming.** 2003. Analysis of an orf virus chemokine-binding protein: shifting ligand specificities among a family of poxvirus viroceptors. Proc. Natl. Acad. Sci. USA **100:**15137–15142.
34. **Yoshimura, A., T. Zimmers, D. Neumann, G. Longmore, Y. Yoshimura, and H. F. Lodish.** 1992. Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. J. Biol. Chem. **267:**11619–11625.