Soluble FasR ligand-binding domain: high-yield production of active fusion and non-fusion recombinant proteins using the baculovirus/insect cell system

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We used the recombinant baculovirus/insect cell system to express two soluble forms of the mouse Fas receptor (mFasR) extracellular domain (ECD): a monomer comprising the entire ligand-binding portion of mFasR followed by a carboxy-terminal hexa-histidine extension aiding purification by immobilized metal affinity chromatography and an immunoadhesin in which the same 148 residues were fused to the Fc portion of a truncated human IgG1 immunoglobulin heavy chain. Both constructs harboured a 24 base pairs insertion placed upstream of the initiating ATG [Peakman, Charles, Sydenham, Gewert, Page, and Makoff (1992) *Nucleic Acids Res.* **20**, 6111–6112]. Despite its hexa-histidine extension, the monovalent recombinant protein from crude culture media failed to bind immobilized Ni²⁺ unless proteins were first precipitated twice by ammonium sulphate.

The overall procedure then yielded ≈ 10 mg/l of protein which could be purified to near homogeneity using two additional chromatographic steps. The glycosylated polypeptide migrated as a band of $M_r = (21\text{--}31) \times 10^3$ in SDS/PAGE and was monomeric in physiological buffers. Under non-reducing conditions, denaturation in 6 M guanidinium chloride was reversible after slow removal of the denaturing agent. The mFasR immunoadhesin was secreted ($\approx 5\text{--}10$ mg/l) as a disulphide-linked homodimer, and endowed with ligand-binding activity since it could bind FasL on the surface of D11S, FasL-expressing cells. When tested for their ability to inhibit FasR-dependent cell lysis, the soluble dimeric immunoadhesin markedly inhibited FasL-mediated cytotoxicity ($IC_{50} \approx 30$ nM), and was ≈ 6 times as effective as its monomeric counterpart.

INTRODUCTION

The Fas (CD95) receptor (FasR) is a 48 kDa trans-membrane protein [1,2] transducing apoptotic signals upon binding of its cytokine ligand, FasL [3], which is expressed in activated T lymphocytes, ovary, heart and lung [4,5]. Its extracellular, ligand-binding domain consists of three ≈ 40 amino-acid-long cysteine-rich repeated motifs with 4–6 cysteine residues at positions which are well conserved, and which define the tumour necrosis factor (TNF) receptor (TNFR) family of proteins [6]. Like TNFR [7] and related apoptosis-transducing receptors, the FasR intracellular domain (ICD) comprises a conserved ‘death domain’ implicated in signal transduction [8]. Similarly, as other members of the TNF family of cytokines [6], FasL (CD95L) is a trimeric type II membrane protein [3,5]. During complex formation, three FasR moieties are thought to bind one FasL trimer in each of the grooves separating two adjacent subunits [9]. The ligand-induced oligomerization of FasR ECDs induces intracellular domain aggregation, which activates the cascade of proteolytic events culminating in apoptosis [10].

The physiological role of the FasL/FasR pathway was first assigned to the regulation of peripheral T-cell homeostasis [5, for review], but its role in the maintenance of immune privilege [11], and of B-cell tolerance [12] have recently been delineated. Moreover, the basic role of the FasR/FasL pair of interacting molecules in the cytotoxicity of virally infected cells [5] explains the vast array of pathological situations in which this system has

recently been implicated. Its role in autoimmunity was first recognized [13] as the lupus-like phenotypes characterizing spontaneous mutations in mice [14], were correlated with defects in either the FasR [13,15] or FasL [16] genes. Recent work has extended its implication to areas such as graft versus host disease [17,18], autoimmune diabetes [19], escape of some tumour cells from immune surveillance [20,21], HIV infection [22,23] and liver pathology [24,25]. Thus, the major role of the FasL/FasR pathway in the pathogenesis of numerous human diseases emphasizes the need for the efficient production in large amounts of soluble versions of the Fas receptor (or derivatives thereof) which can be used to evaluate their capacity to counteract FasL-induced tissue damage *in vivo*.

Due to the existence of very late genes expressed at high levels in the life cycle of baculoviruses, the lytic recombinant baculovirus/insect cell expression system has become the method of choice for the expression of mammalian proteins [26]. Typically in this system, a recombinant virus harbouring the cDNA of interest produces, upon infection of permissive lepidopteran culture cells or larvae, large quantities of recombinant proteins that are authentically folded, post-translationally modified, and biologically active [27]. Using this system, we report here the production of two soluble versions of mouse (m)FasR in milligram amounts and their purification from post-infection culture media. The dimeric form consists of a chimeric homodimer in which the 148 residues of the entire ligand-binding domain of mFasR were fused to a truncated human IgG1 heavy chain. The non-fusion

Abbreviations used: AcMNPV, *Autographa californica* multicapsid nuclear polyhedrosis virus; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; mAb, monoclonal antibody; PVP, polyvinylpyrrolidone; IMAC, immobilized metal affinity chromatography; BVIC system, baculovirus/insect cell system; ECD, extracellular domain; mFasR, mouse Fas receptor; CAPS, 3-cyclohexylamino-1-propane sulphonic acid; *polh*, polyhedrin-encoding gene; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline (171 mM NaCl, 3.4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄); TTBS, Tris-buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl); GdmCl, guanidinium chloride; E:T ratio, effector to target cell ratio.

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form, which contains the same 148 residues fused to a hexahistidine C-terminal extension and can be purified by immobilized metal affinity chromatography (IMAC) after two sequential $(\text{NH}_4)_2\text{SO}_4$ precipitations, exists as a monomer in physiological buffers and is resistant to 6 M guanidinium chloride (GdmCl) denaturation under non-reducing conditions. Both soluble receptor proteins were endowed with biological activity when tested for their ability to inhibit FasR-dependent cell lysis, the soluble mFas receptor dimer being more effective at blocking D11S-mediated cytotoxicity than its monomeric counterpart.

MATERIALS AND METHODS

Reagents and materials

All chemicals were analytical grade. The wild-type baculovirus *AcMNPV-E2* was provided by Dr. M. Summers (Texas A & M). The biotinylated Jo2 hamster anti-mouse FasR mAb was a generous gift from Dr. Nagata. *p*-Nitrophenyl phosphate (pNPP) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium were from Aldrich (Steinham, Germany). Chromatographic resins were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), except for NTA-agarose which was from Qiagen GmbH (Germany). Cell-culture products were from Gibco (Paisley, Scotland, U.K.). Phycoerythrin-conjugated Streptavidin was from Amersham (Little Chalfont, U.K.). Pluronic F68 was from Serva (Heidelberg, Germany). DOTAP was from Boehringer-Mannheim (Germany). *N*-Glycanase was from Genzyme.

Cell culture and virus

D11S cells [28], L1210-3 and L1210-Fas cells [29] were kindly provided by Dr. P. Golstein and grown in RPMI containing 5 % heat-inactivated foetal bovine serum, glutamate and sodium pyruvate at 37 °C. FasL-dependent lysis of Fas-expressing targets was assessed after incubation of D11S cells with sodium ^{51}Cr chromate-labelled Fas expressing target cells at various effector to target cell (E:T) ratios by measuring $^{51}\text{Cr}^{3+}$ release as described [30]. D11S cells were treated with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 2 h before and during the cytotoxicity assay.

Monolayer cultures of the Sf9 cell line from *Spodoptera frugiperda* (ATCC CRL1711) were maintained at 27 °C in TC100 medium supplemented with 0.33 % Yeastolate (Difco), containing 4 % heat inactivated foetal bovine serum. Clone purified recombinant viruses, generated by co-transfection using lipofection [31] with DOTAP as cationic liposome, were identified by hybridization screening of alkali-transferred micro culture DNA onto nylon replica filter to a ^{32}P -labelled insert-specific probe, as described [32]. The *Trichopulsia ni* cell line BTI 5B1-4 (Hi-five, Invitrogen), was adapted to suspension culture in aerated spinner flasks and cultured in IPL41 medium supplemented with 0.33 % Yeastolate, containing 4 % heat inactivated foetal bovine serum, and 0.1 % Pluronic F68, was used for large-scale protein production in a 72 h infection period.

Construction of recombinant transfer plasmids

All plasmids were constructed and purified according to standard recombinant DNA techniques [33]. A polyhedrin-encoding gene (*polh*)-based transfer vector enabling oligonucleotide-directed mutagenesis was first constructed by introducing an M13 replication origin into pVL1392 (Invitrogen). The degraded ends of *Sa*I-cleaved pVL1392 plasmid were filled in with *E. coli* DNA

polymerase I Klenow fragment and the 2.9 kbp DNA fragment was blunt-end ligated to the 2.6 kbp, M13 replication origin containing DNA fragment resulting from *Pvu*II cleavage of Bluescript SK⁻ plasmid (Stratagene). An appropriate recombinant plasmid was selected and designated pCS1392 (Figure 1). The coding sequence of part of the hinge region and the C_H2 and C_H3 domains of human IgG1 heavy chain C region (amino acid residues 214–443) [34] was then cloned into pCS1392. A PCR fragment was synthesized using as a template a plasmid harbouring the cDNA encoding the Fc portion of human IgG1 [35], and the following pair of primers: 5'-GggccgtggggccAGTCTTGTGACAAACTCACACATGCCC-3', and 5'-GCCgtaccTTATTATTTACCCGAGACAGGGAGAGGCTCTTCTGCGT-3', harbouring a *Sfi*I and a *Kpn*I site (lower case) as sense and anti-sense primer, respectively. The purified 718 bp fragment was digested with *Kpn*I, ligated into *Sma*I and *Kpn*I cleaved pCS1392 and used to transform electrocompetent *E. coli* hosts. *Sma*I mapping and nucleotide sequencing led to the selection of a recombinant plasmid designated pCS1392-Fc-IgG1 (Figure 1).

To generate the mFasR immunoadhesin, the 5' portion of the mouse FasR cDNA encoding its entire extracellular domain (ECD) was fused in frame to the 3' portion of the human IgG1 cDNA by cloning a PCR fragment comprising the entire coding sequence of mFasR ECD preceded by a 24 bp insertion [36] into the pCS1392-Fc-IgG1 transfer vector. In this reaction, a plasmid containing the entire mouse cDNA [4] was used as a template with the following oligonucleotides: 5'-GCTATAgcgccgcTAATCATCCACAGGAGACTTTCTGATGCTGTGGATCTGGGCTGTCCTGCC3' and 5'-GACTggccccacggccTTCGATGGGACTTTGTTTCCTGCAGTTTGTATTGCTG3' as a sense and anti-sense primer, encompassing a *Not*I and a *Sfi*I restriction site (lower-case), respectively. Underlined are (i) the 24 bp insertion upstream of the initiating ATG [36] in the sense primer and (ii) the sequence encoding a protease cleavage site for Factor Xa (Ile-Glu-Gly-Arg) in the antisense primer. The 558 bp fragment obtained was digested with *Not*I and *Sfi*I and ligated into the *Not*I and *Sfi*I cleaved pCS1392-FcIgG1 vector and used to transform electrocompetent *E. coli* hosts. A plasmid, selected by restriction mapping and nucleotide sequencing, designated pCS1392-mFas-FcIgG1 (Figure 1) was cotransfected into Sf9 cells to generate a corresponding recombinant baculovirus.

To produce an unfused mFasR ECD with an hexahistidine C-terminal extension, a PCR fragment was synthesized using 5'-GCTATAgcgccgcTAATCATCCACAGGAGACTTTCTGATGCTGTGGATCTGGGCTGTCCTGCC-3' and 5'TTATCTGTGATGGTGTGATGGTGTGCGATTCTGGGACTTTGTTTCCTGC3' as sense, comprising a *Not*I restriction site (lower case) and the 24 bp insertion upstream of the initiating ATG (underlined), and as anti-sense primer comprising the hexa-histidine extension (underlined), followed by a TAA terminating codon, respectively. The 569 bp PCR fragment was cleaved with *Not*I and ligated into *Not*I and *Sma*I digested pCS1392 transfer vector, and used to transform electrocompetent *E. coli* hosts. A recombinant plasmid, selected by restriction mapping and nucleotide sequencing, designated pCS1392-mFas-H6 (Figure 1), was cotransfected into Sf9 cells to generate a corresponding recombinant baculovirus.

PAGE analysis and immunoblots

Protein samples were analysed after boiling in SDS-containing buffer by SDS/PAGE on 10 % polyacrylamide gels using Tris-Tricine buffer [37], and stained with Coomassie Brilliant blue. Proteins were electrotransferred after electrophoresis onto PVDF membranes (Immobilon Millipore) in 10 mM 3-cyclohexyl-

amino-1-propane sulphonic acid buffer (CAPS) pH 11.0, 15 % methanol as anode buffer, and 10 mM CAPS, 15 % methanol, 0.1 % SDS as a cathode buffer, after the gel had been equilibrated for 5 min in cathode buffer. Membranes were blocked in TTBS (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20) containing 1 % polyvinylpyrrolidone (PVP) and incubated with antiserum in TTBS containing 0.1 % PVP, washed with the same solution, and developed using an appropriate alkaline phosphatase-conjugated second antibody (Promega U.S.A.). Goat anti-human Fc polyclonal antibodies were from Biosys (France).

Purification of recombinant proteins from post-infection culture medium

Medium conditioned for 72 h by $(1-3) \times 10^9$ recombinant baculovirus-infected cells was clarified by low speed centrifugation. Immunoadhesin (mFas-FcIgG1) was purified, after concentration by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 60 % saturation. The pellet was redissolved in 50 mM Tris/HCl pH 8.0, 500 mM NaCl, 0.05 % Tween-20, clarified by centrifugation prior to incubation for 1 h at 4 °C with 1.5 ml of Protein A-Sepharose previously equilibrated in the same buffer. Beads were collected by sedimentation and washed three times in equilibration buffer. The bound protein was eluted with 2-3 bed volumes of 100 mM sodium citrate, pH 3.5, and immediately neutralized by an appropriate volume of 2.0 M Tris/HCl pH 9.

mFas-H6 was purified by IMAC after two sequential precipitations of the bulk of soluble protein by $(\text{NH}_4)_2\text{SO}_4$ at 90 % saturation. Crude culture supernatant was brought to 90 % saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and kept at 4 °C for 1 h. The pellet was redissolved in 1/4 volume of 25 mM Tris/HCl, pH 7.5, and the precipitation step with $(\text{NH}_4)_2\text{SO}_4$ at 90 % saturation was repeated. After redissolution of the precipitate in N-buffer (50 mM Hepes pH 7.5, 0.5 M NaCl, 2 % Tween 80) containing 7.5 mM imidazole and clarification, 1-2 ml of NTA-agarose resin saturated with Ni^{2+} , previously equilibrated in the same buffer was added to the protein solution and stirred for 1 h at 4 °C. Beads were collected in a small column and washed with 10 bed volumes of N-buffer followed by 5 bed volumes of N-buffer containing 25 mM imidazole and finally eluted with 2-3 bed volumes of N-buffer containing 125 mM imidazole. Eluted protein was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 90 % saturation and kept at 4 °C.

For hydrophobic interaction chromatography, samples were dissolved in 25 mM Tris/HCl pH 7.5, 1.0 M $(\text{NH}_4)_2\text{SO}_4$ and applied to a 1 ml butyl-Sepharose column equilibrated in the same buffer. The flow-through was collected and desalted on a PD10 column in 25 mM Tris/HCl, pH 8.0, 10 mM NaCl and applied to a MonoQ ion-exchange column equilibrated in the same buffer. N-terminal sequence analysis of purified mFasR was performed by automated Edman degradation after electrophoresis on SDS/PAGE and transfer onto PVDF membranes as described [38], on an Applied Biosystems 470 gas-phase peptide sequencer. Phenylthiohydantoin amino acids were detected with an on-line Applied Biosystems 120 A analyser. Amino acid composition analysis was carried out after total hydrolysis in 6 M HCl at 110 °C for 24 h on a Beckman 6300 amino acid analyser. Hexosamine composition determination was carried out similarly after partial hydrolysis at 100 °C in 4 M HCl for 4 h.

Biochemical methods

Protein concentrations were determined either using the dye binding assay [39] or, for pure proteins, from A_{278} determinations [40].

For *N*-glycosylation studies, purified mFasR-H6 protein was denatured in 50 mM phosphate buffer (pH 6.0) containing 0.1 % SDS and NP40 was added in a seven-fold molar excess with respect to SDS, prior to addition of *N*-glycanase. After 18 h incubation at 37 °C, control (mock) and enzyme-containing samples, were diluted in SDS sample buffer under reducing conditions, analysed on 10 % SDS/PAGE and stained with Coomassie Brilliant Blue.

For renaturation studies, partially purified mFas-H6 protein was denatured in 50 mM Hepes pH 7.5 buffer, containing 6 M GdmCl, and bound onto Ni-NTA-agarose resin equilibrated in the same buffer. After washing with several column volumes, a linear gradient of 6-0 M GdmCl was applied to the column while protein was bound to it over a 1 h period. Soluble protein was eluted with 125 mM imidazole in 50 mM Hepes pH 7.5, dialysed against PBS and assayed for Jo2 binding activity.

mFas antiserum and immunoassays

To prepare anti-mFas polyclonal antibodies, rabbits were injected with 50 µg of pure mFas-H6, in complete Freund's adjuvant. Prior to serum collection, two booster injections in incomplete Freund's adjuvant were given at 3 week intervals. Chicken antibodies were produced by Agro-bio (Villeny, France) by injecting 100 µg of purified mFas-H6 emulsified with complete Freund's adjuvant on days 0, 10 and 21 and sera were collected on day 35 post-immunization. Immune recognition of the purified mFasR by these polyclonal antiserum was assessed by ELISA. Briefly, microtitre plates were coated with purified mFas-H6 before being saturated within TTBS-1 % PVP. Serial dilutions of sera in TTBS-0.1 % PVP were then added and incubated overnight at 4 °C. After washing with TTBS-0.1 % PVP, chicken antibodies were revealed by a 1 h incubation at 4 °C with an anti-chicken Ig rabbit antiserum and all wells were revealed by a final incubation (1 h at 4 °C) with an alkaline phosphatase-conjugated anti-rabbit Igs (Promega). After three final washes in TBS, residual alkaline phosphatase activity was determined after substrate addition by A_{405} readings on a microplate spectrophotometer.

A competition assay with the membrane-bound mFasR expressed on the surface of L1210-Fas cells for Jo2 mAb binding was used to assess the structural integrity of denatured recombinant protein. Briefly, a sub-saturating concentration of biotinylated Jo2 mAb in phosphate-buffered saline (PBS) was incubated with 100 ng of purified mFas-H6 for 2 h at 4 °C and residual antibody binding to L1210-Fas cells expressing membrane-bound mFasR on their surface was evaluated by immunofluorescence.

For flow cytometry, viable cell suspensions were first dispersed in PBS containing 4 % foetal bovine serum and 0.1 % NaN_3 and divided into aliquots of 10^5 cells per tube. Upon incubation at 4 °C for 30 min with primary reagent, cells were washed and further incubated with a saturating concentration of fluorescent Streptavidin conjugate as a developing reagent for 30 min. Cell fluorescence was analysed on a flow cytometer (FacScan, Becton-Dickinson) and fluorescence histograms emitted from single viable cells were generated. Mean fluorescence was recorded for each data point.

RESULTS

High levels of mFas-H6 purified from culture media by the $(\text{NH}_4)_2\text{SO}_4$ /IMAC procedure

The pCS-mFas-H6 transfer plasmid encoding the ligand-binding domain of mouse FasR fused in frame to a hexa-histidine C-

Figure 1 Construction of the modified *polh*-based transfer vector pCS-FcIgG1 and of the recombinant plasmids pCS-mFas-H6 and pCS-mFas-FcIgG1 harbouring the truncated mouse FasR cDNA

The 5.5 kbp pCS1392 replacement vector derives from pVL1392 by addition of the M13⁻ replication origin (dark stippled arrow). The truncated cDNA encoding part of the hinge region and the Fc fragment of the human IgG1 (open arrow) was first cloned into pCS1392 as described in the Materials and methods section, yielding the pCS1392-FcIgG1 transfer vector. The gene fragment encoding the truncated mFasR ECD (dark filled segment) was then cloned either in the pCS1392 transfer vector or in the pCS1392-FcIgG1 yielding pCS-mFas-H6 and pCS-mFas-FcIgG1 respectively. Both of these vectors were subsequently used to target the truncated mouse Fas cDNA to the *polh* locus of *AcMNPV*.

terminal sequence and comprising the 24 bp DNA sequence upstream of its initiating ATG (Figure 1) was used to generate, by homologous recombination, a recombinant virus harbouring the mFas-H6 cDNA at the *polh* locus. Since the mFas receptor fragment (mFas-H6) should be secreted into the culture medium, we set out to purify the His-tagged protein by Ni²⁺-IMAC starting from post-infection culture supernatant. Surprisingly, no mFas-H6 protein could be recovered by IMAC in crude culture media from recombinant virus-infected cells. However, if the bulk of soluble proteins were first precipitated by (NH₄)₂SO₄ at 90 % saturation prior to IMAC, 0.4 mg of mFas-H6 protein could be recovered per litre of culture medium. Remarkably, this amount increased $\approx$ 25-fold (10 mg/l culture medium) upon subsequent re-precipitation with (NH₄)₂SO₄. Further precipitations did not change this yield. This result suggests that low molecular mass molecules preventing metal/His-tagged protein interactions are initially present in the culture medium and are subsequently separated from the bulk of proteins by two sequential (NH₄)₂SO₄ precipitations.

The protein recovered by this (NH₄)₂SO₄/IMAC procedure was obtained with high yield (10 mg/l) and was $\approx$ 75 % pure as judged by SDS/PAGE analysis (result not shown). The mFas-H6 protein was further purified by hydrophobic interaction and ion-exchange chromatography. This three steps purification procedure yielded an essentially pure protein migrating as a wide

Figure 2 SDS/PAGE analysis of purified mFas-H6

A three-step purification scheme starting from post-infection supernatant was carried out by the ammonium sulphate/IMAC procedure, followed by hydrophobic interaction and ion-exchange chromatography. 2.5 μ g of mFas-H6 purified protein was analysed (A). *N*-glycanase digestion of purified mFas-H6: Purified mFas-H6 protein was either mock-digested (B) or digested by *N*-glycanase (C) for 18 h at 37 °C. All samples were diluted in SDS sample buffer under reducing conditions, analysed on 10 % SDS/PAGE and stained with Coomassie Brilliant Blue.

band of apparent relative molecular mass (M_r) of $(21-31) \times 10^3$ when analysed by SDS/PAGE, as shown in Figure 2. The identity of this protein band with the mFas-H6 ECD was substantiated by partial N-terminal sequencing and aminoacid composition analysis which showed that the protein had the expected composition (results not shown). Thus, the recombinant mFas-H6 virus produced high amount of soluble mFasR ECD which could be purified with high yield using (NH₄)₂SO₄/IMAC procedure as a first step.

Biochemical and immunological characterization of mFasR-H6

Under reducing conditions, the observed electrophoretic mobility of the recombinant protein was slower [apparent M_r $(21-31) \times 10^3$] than that expected from the length of the polypeptide chain (154 amino acids M_r 17.4×10^3). Since the mFasR protein sequence comprises two putative Asn-linked *N*-glycosylation sites in its ECD, the extent to which these post-translational modification sites were utilized was assessed by *N*-glycanase digestion of the recombinant protein. As shown in Figure 2, *N*-glycanase digestion decreased the apparent molecular mass of the mFas-H6 protein, indicating that recombinant mFas-H6 is indeed *N*-glycosylated, a contention further supported by hexosamine analysis which revealed the presence of 2.3 residues/molecule of *N*-acetyl glucosamine.

In view of the fact that FasR ECD oligomerization initiates a cascade of events culminating in cell death, one might speculate that some mechanisms operate to prevent its ligand-independent aggregation. It was thus of interest to examine the status of mFasR ligand-binding domain in physiological solutions. Partly

Figure 3 Gel permeation chromatography of purified mFas-H6 protein

50 μ g of partly purified mFas-H6 protein was dissolved in 25 mM Tris/HCl pH 7.5, 150 mM NaCl and applied onto a 500 μ l Superdex column equilibrated in the same buffer. The column was developed with the same buffer on a Smart System (Pharmacia) and A_{278} was monitored (A). 50 μ l fractions were collected and protein contents of peaks a and b were analysed by SDS/PAGE after precipitation with 15% TCA followed by Coomassie Brilliant Blue staining (B).

Figure 4 Subunit structure and antigenicity of the mFas-FcIgG1 immunoadhesin

mFas-FcIgG1 immunoadhesin (2.5 μ g), purified from post-infection supernatant by affinity chromatography on Protein A-Sepharose was analysed by SDS/PAGE under reducing (A) or non-reducing (B) conditions and stained with Coomassie Brilliant Blue. For immunoblot analysis, mFas-H6 (C and E) or mFas-FcIgG1 immunoadhesin (D and F) were separated by SDS/PAGE prior to electrotransfer and membranes were incubated with anti-mFas (C and D) or anti-human Fc polyclonal antibody (E and F). Immunoblots were developed using alkaline phosphatase-conjugated anti-rabbit IgG antibodies.

Table 1 Effect of a GdmCl denaturation–renaturation cycle under non-reducing conditions on mFas-H6 Jo2 immunoreactivity

500 μ g of purified mFas-H6 was denatured in 50 mM Hepes pH 7.5 containing 6 M GdmCl and allowed to bind to a 0.5 ml Ni-NTA-agarose column equilibrated in the same buffer. The chaotrope was then slowly removed over a period of 1.0 h by running a 6 to 0 M GdmCl linear gradient. Renatured protein was then compared with native mFas-H6 for its ability to bind Jo2, an anti-mouse Fas mAb, 100 ng of biotinylated Jo2 was preincubated with a 5-fold molar excess of either native or renatured mFas-H6 protein and the resulting mixture was tested for its residual capacity to bind mFasR on the surface of L1210 cells transfected with the mFasR cDNA. Cell surfaces were then labelled by incubation with PE-conjugated phycoerythrin. Single cell fluorescence was measured by flow cytometry.

Competitor (mFas-H6)	Mean Jo2 fluorescence (A. U.)	Relative Jo2 fluorescence (%)
None	80	100
Native	7	9
Renatured	12	15

purified mFas-H6 was therefore analysed by gel permeation chromatography in 25 mM Tris/HCl pH 7.5, 150 mM NaCl. As shown in Figure 3, two A_{278} peaks could be detected: a minor peak (a) eluting soon after the void volume (M_r 450 $\times$ 10³), and a major peak (b) corresponding to a M_r 25 $\times$ 10³. Analysis of the protein contents of these two peaks by SDS/PAGE revealed that all of the mFas-H6 protein was present in the major peak, the minor peak containing a contaminating protein of M_r 50 $\times$ 10³. These data shows that in solution in physiological buffers, mFas ECD is strictly monomeric.

To gain insight into the immunological properties of mFasR, purified mFas-H6 was used to raise antibodies in rabbit and chicken. As expected, the rabbit antiserum was able to recognise mFasR on the surface of L1210 FasR-transfected cells and of CD4⁺CD8⁺ mouse thymocytes (results not shown). In addition, this antiserum strongly reacted with FasR in immunoblots (Figure 4). Chicken antibodies also recognized mFasR in immunoblots, albeit with lower intensity (result not shown). In addition, both rabbit and chicken antiserum recognized mFas in ELISA (not shown).

mFas-H6 conformation is resistant to GdmCl denaturation

To gain further insight into the biochemical properties of soluble mFasR ligand-binding domain, mFas-H6 was submitted to denaturation in 6 M GdmCl in neutral buffer under non-reducing conditions to preserve the integrity of the disulphide bridges present in the protein structure. Purified mFas-H6 protein was first denatured in 6 M GdmCl and then bound to a Ni-NTA-agarose column. While column-bound, the protein was allowed to renature by applying a 6–0 M GdmCl linear gradient over a 1 h period and eluted in buffer containing 125 mM imidazole. Thus 80% of the input protein could be recovered, indicating that most of the protein was soluble after complete removal of GdmCl. To assess the conformational integrity of the renatured protein, its recognition by Jo2, a hamster anti-mouse FasR monoclonal antibody [24], was compared to that of the native protein in a surface labelling inhibition assay. As shown in Table 1, renatured mFas-H6 was almost as effective at inhibiting Jo2 binding to surface mFasR as its native counterpart, indicating that the Jo2 epitope had been recovered in the denaturation/renaturation process.

Figure 5 mFas-FcIgG1 binds FasL on the surface of D11S cells

Living D11S cells were incubated with either mFas-FcIgG1 (thick line) or buffer alone and both samples were further incubated with biotinylated anti-human IgG antibody. After extensive washings in PBS, cells were last incubated with FITC-conjugated streptavidin and surface fluorescence of undamaged individual cells was measured by flow cytometry.

Figure 6 Soluble mFasR proteins inhibit FasL-induced D11S cell cytotoxicity

Parental and Fas-transfected L1210 cells were loaded with $^{51}\text{Cr}^{3+}$ and incubated with D11S cells at a 10/1 effector/target ratio, in the presence of various concentrations of either mFas-H6 (open circles) or mFas-FcIgG1 (closed circles) soluble recombinant protein. After 12 h, $^{51}\text{Cr}^{3+}$ release was determined. To allow comparisons between molecules of different valencies, concentrations given above are of the monomer units, i.e. of the FasR moiety. Control immunoadhesin was ineffective (see text).

mFas-FcIgG1 is produced as a functional disulphide-linked chimeric homodimer

mFas immunoadhesin was created by fusing complementary DNAs encoding the extracellular portion (amino acids 1–145) of the murine FasR and the hinge and constant regions $\text{C}_{\text{H}2}$ and $\text{C}_{\text{H}3}$ domains (228 residues, amino acids 214–443) of human IgG1 heavy chain separated by a 7-amino-acids linker comprising a factor Xa cleavage site (IEGRGGQ). The total length of the chimeric polypeptide was 381 residues. A plasmid encoding the mFasR immunoadhesin was constructed in two steps. First, a DNA fragment encoding part of the hinge region and the Fc portion of the human IgG1 preceded by a *Sfi*I cloning site was inserted into the pCS1392 baculovirus transfer vector. The resulting vector was designated pCS-FcIgG1 and is depicted in Figure 1. Second, a DNA fragment encoding the ECD of mFasR

cDNA was subcloned into the pCS-FcIgG1 vector, yielding a recombinant plasmid designated pCS-mFas-FcIgG1 (Figure 1) used to generate a recombinant virus encoding the mFas immunoadhesin. Protein A–Sepharose affinity chromatography allowed the recovery of $\approx 5 \text{ mg/l}$ of a nearly pure protein from recombinant virus culture supernatant, migrating as a M_r 50×10^3 polypeptide under reducing conditions (Figure 4, lane A). Under non-reducing electrophoretic conditions, the main protein band migrated with a M_r of $\approx 100 \times 10^3$ (Figure 4, lane B). This decrease in electrophoretic mobility is consistent with a disulphide-linked homodimeric structure, which is that predicted for the mFas-FcIgG1 immunoadhesin. Furthermore, immunoblot analysis revealed that, in addition to being recognized by anti-human Fc antibodies (Figure 4, lane F) the M_r 50×10^3 polypeptide was also recognized by the anti-mFasR antibodies (Figure 4, lane D), whereas the unfused control mFas-H6 protein was recognized by the anti m-Fas antiserum, only (Figure 4, lanes C and E). The minor bands in lanes D and F correspond to residual dimers. This dual recognition confirms the chimeric nature of the immunoadhesin homodimer.

To assess the functional character of the recombinant protein, we tested the capacity of the mFas immunoadhesin to bind the FasL membrane protein present on the surface of the D11S cell line by flow cytometry [3]. When mFas-FcIgG1 was used as a primary reagent, a 10-fold increase in viable cell surface mean fluorescence was observed (Figure 5), indicating that the mFas immunoadhesin produced in the baculovirus/insect cell (BVIC) system was indeed able to bind its ligand FasL. Thus, BVIC system produced a functional immunoadhesin with high yield.

Dimeric mFasR is most effective at inhibiting FasL-mediated cell lysis

Since mFas-FcIgG1 was able to bind FasL, its ability to inhibit FasL-mediated cytotoxicity was tested and compared with that of its monomeric counterpart in an assay where lysis of Fas-expressing target cells by D11S was monitored. As shown in Figure 6, addition of dimeric mFas-FcIgG1 to the culture media markedly inhibited FasR-specific lysis in a dose-dependent manner with a half-maximal effective concentration (IC_{50}) of $\approx 50 \text{ nM}$. In contrast, as previously observed with mouse thymocytes [41], a control immunoadhesin in which human placental alkaline phosphatase replaced the mFas ECD was ineffective (result not shown). Monomeric mFas-H6 was much less effective at inhibiting D11S-induced cell lysis and its half-maximal effective concentration was $\approx 250 \text{ nM}$. Thus, both recombinant proteins were active at inhibiting FasL-induced lysis of FasR-bearing target cells but the divalent immunoglobulin-like form was ≈ 6 times more effective in this respect than its monovalent counterpart.

DISCUSSION

The BVIC system has proven useful for overexpressing a number of membrane receptors [42,43], some of them as truncated versions secreted into the culture medium [44,45]. To facilitate purification of recombinant proteins by IMAC on resins coupled with chelating agents [46], hexa-histidine extensions inserted at either end of recombinant proteins have been widely used both in prokaryotic [47] and eukaryotic [48] expression systems. These premises prompted us to insert at the C-terminal end of the truncated mouse FasR receptor ECD an hexa-histidine extension and express the corresponding recombinant protein in the BVIC system. Our first attempts to purify the secreted His-tagged FasR

ECD by IMAC have been hampered by the lack of any specific binding of the recombinant protein present in crude culture media to Ni-NTA-agarose. We reasoned that this failure could be attributed to the presence in the culture medium of one or several interfering molecule(s) and have thus designed a simple biochemical method to separate them from the soluble His-tagged recombinant protein. In support of this view was the observed stepwise increase in protein recovery which became maximal after the second $(\text{NH}_4)_2\text{SO}_4$ precipitation. We have not attempted to characterize the interfering compound(s) but among possible candidates stand, besides free histidine, amino acids such as glycine, cysteine, lysine or arginine or other substances such as metal ions. Even though their respective concentrations in the defined components of insect cell culture media seem too low to account for the observed effect, some of them may increase substantially upon addition of media supplements whose composition is ill-defined (Yeastolate, serum). The purification method described here is relatively simple and inexpensive, has been used with success for several other His-tagged recombinant proteins expressed in the BVIC system (J. Mahiou et al. unpublished work) and should have wide applications for the non-denaturing purification of any secreted His-tagged recombinant protein produced using the BVIC system and also other expression systems using rich cell culture media. The amount of mFas-H6 present in the culture medium was high considering the low efficiency of secretion reported for the BVIC [49]. Due to the lack of reliable assay, no evaluation of this process was attempted here, but secretion of mFas-H6 was certainly incomplete since recombinant protein could also be extracted from infected cell homogenates (F. Godeau, unpublished work). Furthermore, $\approx 60\%$ of total enzyme activity was secreted in the culture medium of cells infected with a baculovirus encoding a soluble version of alkaline phosphatase (SeAP) (F. Godeau, unpublished work).

The purification of soluble mouse FasR ECD to near homogeneity, which constitutes a potential starting point for future crystallization studies, was obtained with the use of two additional chromatographic steps. The chromatographic behaviour of mFas-H6 ECD on butyl-Sepharose indicates that the soluble FasR ligand-binding domain is hydrophilic, as it does not bind the hydrophobic resin even in the presence of 1 M $(\text{NH}_4)_2\text{SO}_4$. Furthermore, the lack of binding of the recombinant protein to a MonoQ column at pH 8.0 in low salt buffer is consistent with a pI of 8.6, a value predicted from the amino acid composition of the polypeptide [50]. The pure protein migrated as a wide band in SDS/PAGE with a M_r higher than that of the corresponding polypeptide. *N*-Glycosylation might account for this discrepancy, but whereas the recombinant protein is indeed *N*-glycosylated, *N*-glycanase-digested recombinant protein still migrates as a wide band with a $M_r > 17.4 \times 10^3$. The reason for this behaviour is not clear at present and needs to be further investigated.

The availability of purified monovalent FasR recombinant protein in large amounts also enabled us to describe two additional biochemical properties of mFasR ligand-binding domain. The reversibility of its denaturation by chaotropes under non-reducing conditions, even at very acidic pH (result not shown), suggests a major contribution of the covalent disulphide bonds to the establishment and maintenance of mFasR tertiary structure. This contention is further supported by the heat-stability (80 °C for 10 min) of the polypeptide (result not shown). Moreover, we have shown that the soluble form of mFasR ECD remains strictly monomeric and does not undergo any detectable aggregation in physiological buffers. Provided that the presence of the C-terminal hexa-histidine extension does not contribute to the monomeric state of the recombinant molecule, this ob-

servation is consistent with the notion that the uncontrolled aggregation of FasR ECDs in the cell membrane constitutes a potentially deleterious event which should be prevented since it initiates the cascade of events culminating in cell death [10]. Finally, we have also been able to raise antibodies against the mouse FasR ECD which should enable the development of immunoassays aimed at measuring soluble mFasR, a form of potential importance in various pathological conditions [51].

Fusion proteins have been used to study a variety of proteins in a wide array of applications. Particularly useful for secreted proteins undergoing post-translational maturation events during their transit in the secretory pathway, is the immunoglobulin fusion protein, in which the protein of interest is fused to the hinge and Fc portion of an immunoglobulin heavy chain constant region (immunoadhesin) [52]. The fusion protein usually resembles a bivalent antibody without light chains, can be easily purified by affinity chromatography using immobilized Protein A and used for various assays with readily available conjugated anti-Ig reagents [52]. Such Ig fusion proteins are usually produced using transiently or stably transfected mammalian cells such as 293 cells [53], J558L myeloma cells [54] or CHO cells [55]. Even though baculovirus expression of immunoadhesins has already been reported [56,57], we have constructed pCS-FcIgG1, a versatile transfer vector allowing high level expression of future immunoglobulin fusion protein in the BVIC system. This vector, was used in the present study to express the mFasR ECD as an immunoglobulin fusion protein. The mFas-immunoadhesin mFas-FcIgG1 is produced in large quantities as a disulphide-linked homodimer, as reported for immunoadhesins expressed in other systems [52]. Its biological activity was demonstrated by its ability to stain the surface of FasL-bearing D11S cells specifically and inhibit their cytotoxicity towards FasR-bearing target cells. In this respect, the observed properties of insect cells-produced mFasR-Ig are similar to those of another Fas-immunoadhesin produced using myeloma cells [3].

Our *in vitro* studies comparing the biological activity of the monomeric and dimeric forms of truncated mFasR indicate that mFas-FcIgG1 is ≈ 6 times more effective at inhibiting FasL-induced cytotoxicity than mFas-H6. Similarly, when the effect of valency on the inhibitory activity has been assessed, the bivalent Ig fusion protein competed at least one order of magnitude more efficiently than the monovalent receptor fragment. Thus, a difference of 24, 20 and 200-fold has been reported for divalent TNFR [58], IFN γ R [55] or ICAM1 [59], respectively. The structural properties of the Fas-Fc homodimeric form which give it an advantage over the monomeric form as a FasL antagonist, have not completely been explored but it is likely that the Fas-Fc binds two sites of the FasL trimer, thereby rendering it biologically less active by limiting its ability to interact with several surface receptors. These physical properties will require further study.

The high yield observed in the production of these two soluble proteins may be attributed in part to the insertion in their respective mRNAs of the 24 bp sequence upstream from the initiating ATGs. Although no attempt was made to compare the production efficiency in its absence, this observation is consistent with the fact that this sequence increases the translational efficiency of the mRNA in front of which it has been placed [36]. Since the yield in purified protein obtained using the BVIC expression is higher than that reported for other expression systems, the potential control, by injections of these kind of molecules, of pathological conditions which involve the immunosuppressive [20], or tissue damaging [17–19] capacities of FasL becomes amenable to *in vivo* studies. This should help to evaluate their therapeutic potential.

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