Protein Expression and Purification 94 (2014) 7–14

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Purification and biological characterization of soluble, recombinant mouse IFNβ expressed in insect cells

Sebastian A. Stifter [a], Jodee A. Gould [a], Niamh E. Mangan [a], Hugh H. Reid [b], Jamie Rossjohn [b,c], Paul J. Hertzog [a], Nicole A. de Weerd [a,*]

[a] Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, Monash University, Clayton, Victoria 3800, Australia
[b] Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria 3800, Australia
[c] Institute of Infection and Immunity, Cardiff University, School of Medicine, Heath Park, Cardiff CF14 4XN, UK

ARTICLE INFO

Article history:
Received 14 September 2013
and in revised form 28 October 2013
Available online 6 November 2013

Keywords:
Interferon beta
Insect cell expression
Soluble protein
Natively folded
Endotoxin-free
Biologically active

ABSTRACT

Interferon β (IFNβ) is a member of the type I interferon family of cytokines widely recognised for their anti-viral, anti-proliferative and immunomodulatory properties. Recombinant, biologically active forms of this cytokine are used clinically for the treatment of multiple sclerosis and in laboratories to study the role of this cytokine in health and disease. Established methods for expression of IFNβ utilise either bacterial systems from which the insoluble recombinant proteins must be refolded, or mammalian expression systems in which large volumes of cell culture are required for recovery of acceptable yields. Utilising the baculovirus expression system and *Trichoplusia ni* (Cabbage Looper) BTI-TN-5B1-4 cell line, we report a reproducible method for production and purification of milligram/litre quantities of biologically active murine IFNβ. Due to the design of our construct and the eukaryotic nature of insect cells, the resulting soluble protein is secreted allowing purification of the Histidine-tagged natively-folded protein from the culture supernatant. The IFNβ purification method described is a two-step process employing immobilised metal-ion affinity chromatography (IMAC) and reverse-phase high performance liquid chromatography (RP-HPLC) that results in production of significantly more purified IFNβ than any other reported eukaryotic-based expression system. Recombinant murine IFNβ produced by this method was natively folded and demonstrated hallmark type I interferon biological effects including antiviral and anti-proliferative activities, and induced genes characteristic of IFNβ activity *in vivo*. Recombinant IFNβ also had specific activity levels exceeding that of the commercially available equivalent. Together, our findings provide a method for production of highly pure, biologically active murine IFNβ.

Introduction

The type I interferons (IFNs)[1] were discovered by virtue of their antiviral activity and have since been shown to also possess anti-proliferative and immunoregulatory functions [1,2]. In humans this multi-gene family comprises 12 IFNα subtypes and individual IFNβ, ε, ω, κ, δ, τ and ζ proteins, that signal via a heterodimeric receptor complex composed of two subunits, IFNAR1 and IFNAR2 [3]. Ligand engagement of the receptor initiates an intracellular signalling cascade involving the JAK-STAT signalling pathway, which subsequently activates numerous interferon stimulated genes (ISGs) resulting in the generation of an immune response [4]. While showing considerable sequence and structural homology across different species, the type I IFNs are highly species specific in regards to receptor interactions and subsequent signalling events.

IFNβ is an inducible component of the host immune response to viral and bacterial infections, and is also used clinically for the treatment of multiple sclerosis [5]. This cytokine is also pivotal to various processes in the body, including lymphoid development, myelopoiesis [6] and the toxicity induced by septic shock [7]. Compared to the IFNα subtypes, IFNβ is also more effective at activating anti-proliferative responses, pro-apoptotic pathways in tumour

* Corresponding author. Tel.: +61 3 99024768.
E-mail addresses: sebastian.stifter@monash.edu (S.A. Stifter), jodee.gould@monash.edu (J.A. Gould), niamh.mangan@monash.edu (N.E. Mangan), hugh.reid@monash.edu (H.H. Reid), jamie.rossjohn@monash.edu (J. Rossjohn), paul.hertzog@monash.edu (P.J. Hertzog), nicole.deweerd@monash.edu (N.A. de Weerd).

[1] *Abbreviations used:* ACN, acetonitrile; CD, circular dichroism; CPE, cytopathic effect; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration (to give 50% effect); EK, enterokinase; EU, endotoxin units; HRP, horseradish peroxidise; IFN, interferon; IMAC, immobilized metal affinity chromatography; ISG, interferon stimulated gene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MWCO, molecular weight cut-off; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real time PCR; RP-HPLC, reverse phase-HPLC; SDS–PAGE, sodium dodecyl sulphate polyacrylamide electrophoresis; SEM, standard error of the mean; Sf, *Spodoptera frugiperda*; TBS, tris buffered saline; TFA, trifluoroacetic acid.

1046-5928/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2013.10.019

cells [8–10] and has a vital role in the control of macrophage differentiation and osteoclastogenesis [11,12]. Recently we established a structural basis for the unique properties of IFNβ [13].

Before the availability of recombinant DNA technology, commercial or laboratory quantities of type I IFNs were extracted from virally infected white blood cells via a single step purification protocol. Thus, early clinical trials used crude protein preparations, in which active IFN was less than 0.1% of the total protein [14]. Based on the acid-stable nature of the type I IFNs, a protocol utilising reverse-phase high performance liquid chromatography (RP-HPLC) was later developed which enabled the production of homogeneous and highly purified preparations of IFN [15]. For the first time, the biological activities of type I IFNs could be precisely demonstrated and characterised, paving the way for future researchers to clearly demonstrate the multipotent activities attributable to these cytokines.

Baculoviral expression systems have been used successfully for the production of biologically-active cytokines including human and canine IFNs [16–18]. These systems are ideal for the production of recombinant mammalian-derived cytokines as, due to their eukaryotic nature, insect cells produce soluble, natively folded proteins. Also, since these cells are not prokaryotic in origin, there is no risk of contaminating endotoxin, an impurity of bacterial expression systems.

To investigate the functions of IFNβ in mouse models and cell culture, requires a high-yielding source of biologically-active, endotoxin-free protein. We therefore developed an insect cell-based expression and purification protocol for the large scale production of highly-purified, endotoxin-free murine IFNβ. The cytokine produced in this system displays comparable levels of *in vitro* and *in vivo* biological activity to a commercial IFNβ, but has a higher specific activity, thus demonstrating greater purity of the functional protein.

Materials and methods

Plasmid construction

The *mIfnb1* gene was amplified from C57BL/6 genomic DNA using *Pfu* polymerase with amplification directed by specific forward (GGATCCTATAAGCAGCTCCAGCTC) and reverse primers (GTTAAGCTTAGTTTTGGAAGTTTCTGGTAAG). IFNβ1 expressed during this study corresponds to residues 24–182 of murine IFNβ (RefSeq Acc No. NP_034640.1). The 490 bp polymerase chain reaction (PCR) product was cloned into a modified pFastBac™1 vector (Life Technologies) and contained the gp67 signal sequence of the *Autographa californica* nucleopolyhedrovirus followed by the His_6 tag, enterokinase (EK) cleavage site and a flexible linker. After transformation of competent JM109 cells (Bioline), transformants were screened for inserts by colony PCR using the *mIfnb1* forward and reverse primers above with positive clones sequenced with the polyhedrin sequencing primer (AAATGATAACCATCTCGC).

Generation of recombinant bacmid and baculoviral stocks

For expression in insect cells using the Bac-to-Bac Baculoviral Expression System (Life Technologies), recombinant bacmids incorporating pFastBac-*mIfnb1* were generated in the AcBacΔCC bacmid [19] following the manufacturer's instructions (Life Technologies). Transformed clones were identified by blue/white colour selection and screened by colony PCR using M13 forward (GTTTTCCCAGTCACGAC) and reverse (CAGGAAACAGCTATGAC) primers. Positive clones were isolated using a Qiagen EndoFree Maxi-Prep kit according to the manufacturer's instructions (Qiagen). Recombinant baculovirus was generated by transient transfection of the recombinant bacmid into *Spodoptera frugiperda* (Sf)-9 insect cells according to the manufacturer's instructions (Life Technologies). To generate high titre viral stocks, the viral supernatants from these transient transfections were amplified in two rounds of low multiplicity of infection (MOI of ~0.1) of 1×10^6 Sf9 cells mL^{-1} for 4–5 days.

Insect and mammalian cell culture and reagents

The mouse L929 fibroblast cell line was purchased from the American Type Culture Collection. Mouse macrophage RAW 264.7 cells were a kind gift from Dr. Ashley Mansell (Monash Institute of Medical Research, Victoria, Australia). Both L929 and RAW 264.7 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (ICP, New Zealand), 1% L-glutamine (Life Technologies) and 1% Penicillin/Streptomycin (Life Technologies) at 37 °C, 5% CO_2. Serum free adapted insect cell lines Sf9 and High Five™ (BTI-TN-5B1-4 from *Trichoplusia ni*) were purchased from Life Technologies and maintained in Sf900-II media (Life Technologies) supplemented with 1 μg mL^{-1} Gentamicin (Sigma–Aldrich) in a shaking incubator at 27 °C, 120 rpm. For expression cultures, High Five™ cells were diluted in serum-free Express Five media (Life Technologies) supplemented with 20 mM L-Glutamine and 1 μg mL^{-1} Gentamicin (Sigma–Aldrich) and incubated as above. Recombinant murine IFNβ (containing 0.1% (w/v) bovine serum albumin) was purchased from Sigma Aldrich. The certificate of analysis for each batch of this reagent details the batch-specific IFN activity (given as international units (IU) mL^{-1}) and its specific activity in relation to IFN content (given as IU mg^{-1} of protein).

Expression of recombinant IFNβ

IFNβ was expressed as a soluble protein secreted into the culture supernatant. For recombinant protein expression, cells at 2×10^6 cells mL^{-1} were infected with the amplified high-titre recombinant IFNβ baculoviral stocks at a ratio of 1 mL virus to 10 mL cells (a multiplicity of infection of ~10). Expression was carried out at 27 °C for 48 h on an orbital shaker rotating at 120 rpm. Cultures were transferred to sterile 50 mL conical falcon tubes (Becton–Dickinson), the cells pelleted by centrifugation at 160 *g* 4 °C for 20 min, followed by a second centrifugation step at 6000 *g*, 4 °C for 20 min. The cell pellet was discarded and the supernatant retained for protein purification.

Purification of recombinant His_6-IFNβ

Soluble His_6-IFNβ was purified from the insect cell culture supernatant. After the addition of the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF; to 1 mM final concentration), the supernatant was dialysed against TBS (10 mM Tris–HCl, 150 mM NaCl, pH8.0) overnight at 4 °C using 12.5 kDa cut-off dialysis tubing (Sigma Aldrich). To reduce non-specific interactions with the resin, 20 mM imidazole was then added to the dialysate which was then recirculated at least three times over Nickel Sepharose FastFlow 6 resin (GE Healthcare Life Sciences) to capture His-tagged proteins. Bound proteins were eluted under native conditions using TBS supplemented with 150 mM Imidazole, pH8.0. Fractions that contained IFNβ were pooled, acidified with 1 μl mL^{-1}, 0.1% (v/v) trifluoroacetic acid (TFA) (Merck), filtered (0.2 μm syringe-driven filter; Sartorius) to remove particulates and then injected onto a Phenomenex Jupiter C5 prep and guard column (Phenomenex) attached to an Agilent 1100 modular RP-HPLC. All recombinant IFNβ proteins were resolved using a linear gradient of Buffer A (0% acetonitrile (ACN), 0.1% TFA, pH2.0) into Buffer B (90% ACN, 0.1% TFA, pH2.0) at 3 mL min^{-1} for 60 min.