Protein Expression and Purification 87 (2013) 27–34

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

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

Unexpected mucin-type *O*-glycosylation and host-specific *N*-glycosylation of human recombinant interleukin-17A expressed in a human kidney cell line

Kieran F. Geoghegan [a,*], Xi Song [a], Lise R. Hoth [a], Xidong Feng [a], Suman Shanker [a], Amira Quazi [b], Deborah P. Luxenberg [b], Jill F. Wright [b], Matthew C. Griffor [a]

[a] *Pfizer Worldwide Research, Groton, CT 06340, United States*
[b] *Pfizer Worldwide Research, Cambridge, MA 02140, United States*

ARTICLE INFO

Article history:
Received 14 August 2012
and in revised form 23 September 2012
Available online 13 October 2012

Keywords:
Interleukin-17A
N-glycosylation
O-glycosylation
Mass spectrometry
Tandem MS
HEK293 cells
Cell-specific glycosylation

ABSTRACT

The T helper cell-derived cytokine interleukin-17A (IL-17A) is a variably glycosylated disulfide-linked homodimer of 34–38 kDa. Its polypeptide monomer contains one canonical *N*-glycosylation site at Asn68, and human recombinant IL-17A was partly *N*-glycosylated when expressed in human kidney (HEK293) cells as a fusion protein with a melittin signal sequence and an N-terminal hexahistidine tag. Orbitrap mass analyses of the tryptic *N*-glycopeptide 63–69 indicated that the *N*-glycosylation was of the GalNAc-terminated type characteristic of cultured kidney cells. The mass spectrum of IL-17A monomer also included peaks shifted by +948 Da from the respective masses of unglycosylated and *N*-glycosylated polypeptides. These were caused by unpredicted partial *O*-glycosylation of Thr26 with the mucin-like structure -GalNAc(-NeuNAc)-Gal-NeuNAc. Identical *O*-glycosylation occurred in commercially sourced recombinant IL-17A also expressed in HEK293 cells but with a different N-terminal sequence. Therefore, the kidney host cell line not only imposed its characteristic pattern of *N*-glycosylation on recombinant IL-17A but additionally created an *O*-glycosylation not known to be present in the T cell-derived cytokine. Mammalian host cell lines for recombinant protein expression generally impose their characteristic patterns of *N*-glycosylation on the product, but this work exemplifies how a host may also unpredictably *O*-glycosylate a protein that is probably not normally *O*-glycosylated.

Introduction

Mammalian host cell lines used for protein expression impose their own specific patterns of *N*-glycosylation on recombinant proteins [1–3]. Detailed studies of the cell-specificity of *N*-glycan structures are well advanced [4–6], but less is known about the cell-specificity of *O*-glycosylation, and *O*-glycosylation is generally less predictable. This note describes unexpected *O*-glycosylation of a human cytokine expressed in kidney cells. The result was observed in two versions of the recombinant product, one expressed in our own laboratories and another from a commercial source.

The protein in question was human interleukin-17A (IL-17A[1]; UniProt accession Q16552), a homodimeric cytokine of 34–38 kDa secreted from T helper cells, and originally named cytotoxic T-lymphocyte-associated antigen 8 (CTLA8) [7–9]. Loss of a 23-residue signal peptide [10,11] shortens the 155 amino-acid gene product to a 132-residue polypeptide containing six cysteinyl residues that form two intrachain and two interchain disulfides [12]. IL-17A is the prototype of a class of proinflammatory cytokines that are targets for therapeutic intervention in rheumatoid arthritis and other autoimmune diseases mediated by T helper 17 cells [13–16]. Interleukin-17F (IL-17F: UniProt accession Q96PD4), which is 55% sequence-identical to IL-17A, exists both as a homodimer and as a heterodimer with IL-17A. Much remains to be learned about the IL-17 family of cytokines and their corresponding receptors [17].

Sequence numbers in this note refer to full-length gene products including their signal peptides; we denote the N-terminal residue of IL-17A as Gly24 and the single canonical site for *N*-glycosylation as Asn68. For recombinant proteins with affinity tags at the N-terminus, sequence numbers for the mature protein match the natural sequence and tag-derived regions are described as N-terminal appendages.

The earliest reports of IL-17A expression in eukaryotic cell lines showed both that it was secreted as a mixture of glycosylated and unglycosylated disulfide-linked homodimeric proteins [8,11], and that this pattern replicated the properties of the T cell-derived "natural" protein [11]. Authors have scrupulously avoided stating that *N*-glycosylation at Asn68 accounts for all of this heterogeneity, but this appears to be the prevalent view. *O*-Glycosylation has never been reported in IL-17A, and we failed to detect it in nonre-

* Corresponding author.
E-mail address: kieran.f.geoghegan@pfizer.com (K.F. Geoghegan).

[1] IL-17A, interleukin-17A; CTLA8, cytotoxic T-lymphocyte-associated antigen 8; IL-17F, Interleukin-17F.

1046-5928/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2012.09.013

combinant protein immunoprecipitated from the conditioned medium of cultured T cells.

Peptide N-glycosidase F converted the upper band of an electrophoretic doublet of IL-17A expressed in *Pichia pastoris* to the lower band [18], but *O*-glycosylation was excluded based on the absence of any effect when the protein was treated with *N*-acetyl neuraminidase and an *O*-glycosidase. This conclusion has become questionable because the enzymes used would not be expected to remove the *O*-mannosyl glycans normally found in *P. pastoris* [19,20]. By design, IL-17A made in *P. pastoris* had Ile20 as its N-terminal residue, with a yeast α-factor secretion signal removed from the recombinant gene product by proteolytic processing. Ile20 is also the N-terminal residue of a commercial preparation of human recombinant IL-17A produced in human 293 cells (Cell Signaling Technology, Danvers, MA).

While studying N-terminally His-tagged human recombinant IL-17A expressed in human kidney cells, we examined mass spectra of disulfide-reduced protein. Unglycosylated protein was detected with the correct mass, in addition to multiple species attributable to *N*-glycosylation of a type recently shown to be characteristic of kidney cells. Initially unexplained was a peak of +948 Da additional mass relative to unglycosylated peptide, and the same modification was also carried by a fraction of the N-glycosylated material. It was ultimately shown that the recombinant protein was partly O-glycosylated at Thr26, a previously unknown modification of this important research reagent.

Materials and methods

Expression and purification of human recombinant IL-17A

Sequential overlapping PCR amplifications yielded a gene encoding a fusion protein composed of a honeybee melittin signal sequence (residues 1–21 of UniProt accession P01501) followed by a Gly-Ser-Gly spacer, a hexahistidine tag and a further Gly-Ser-Gly spacer, all followed by residues 20–155 of IL-17A (UniProt accession Q16552). This was cloned into the *Sal* I and *Eco* RI sites of a vector [21] containing a murine cytomegalovirus promoter followed by a tripartite leader sequence and a hybrid intervening sequence modified to contain a neomycin phosphotransferase II gene.

This form of human recombinant IL-17A was expressed in suspension culture of FreeStyle 293-F cells (Invitrogen Cat#R790–07) following the manufacturer's protocol using FreeStyle Max reagent. Nine liters of conditioned medium was harvested, concentrated 10-fold, and diafiltered against Buffer A (0.04 M Tris HCl, 0.3 M NaCl, 0.02 M imidazole, pH 7.5) using a QuixStand hollow fiber system with a 10 K NMWC UFP-10-C-4X2MA cartridge (GE Healthcare). Diafiltered medium was then loaded onto a 5 ml Ni Sepharose 6 Fast Flow column (GE Healthcare Cat#17–5255-01) at a flow rate of 2 ml/min. The column was washed with 10 column volumes of Buffer A and then eluted with a gradient from 0 to 100% Buffer B (40 mM Tris HCl, 0.3 M NaCl, 0.3 M imidazole, pH 7.5) with 2 ml fractions collected. Fractions containing IL-17A were pooled and stored at −80 °C.

Polypeptide intact mass analyses

Intact masses were measured by LC-MS performed using an AB Sciex QSTAR XL time-of-flight spectrometer. Proteins were eluted from a Vydac C4 column (type 214MS5.310) using a gradient of 1.6–75% acetonitrile in 0.1% formic acid delivered by an Agilent 1100 capillary HPLC pump over 21.5 min at a flow rate of 0.02 ml/min to a TurboIonSpray source at the spectrometer. Positive-ion spectra were acquired in the range *m/z* 400–2000 with accumulation time set to 0.499964 s. Mass spectra over the protein peak were averaged and deconvoluted to a mass distribution result using the Bayesian Protein Reconstruct routine in Analyst QS software.

Peptide mapping liquid chromatography/mass spectrometry

LTQ ion trap mass spectrometer (Thermo Scientific): Initial peptide mapping data were collected by routine methods using capillary reversed-phase HPLC (0.005 ml/min).

LTQ Orbitrap XL: This instrument was used to analyze tryptic digests of recombinant IL-17A, and in particular to collect higher-resolution spectra (resolution = 15000) of *N*- and *O*-glycopeptides. MS^n experiments were performed to elucidate glycopeptide and peptide structures. The spectrometer was interfaced with an Agilent 1100 capillary HPLC system operating at a flow rate of 10 μl/min with solvents as follows: A, 0.2% formic acid; B, 0.2% formic acid in acetonitrile. Chromatography was performed using a PROTO 300 C18 5 μm column (10 cm × 0.5 mm; p/n RS-10-M5-W185) from Higgins Analytical (Mountain View, CA). The LTQ Orbitrap XL was equipped with a CaptiveSpray interface (Bruker-Michrom).

LTQ Orbitrap Velos: This instrument was used only to analyze in-gel digests of protein in sections from the tricine–SDS polyacrylamide gel fractionation of IL-17A immunoprecipitated from conditioned medium of activated T cells (Supplementary Data Fig. S4). Digests were desalted and freed of particulates using successive steps (precondition, equilibrate, load, wash, elute according to standard reversed-phase HPLC principles) of centrifugally driven liquid flow through 0.2 ml disposable pipet tips packed near the orifice with Empore C18 membrane cutouts, and then dried and redissolved in 0.02 ml of 0.1% formic acid. LC fractionation at a flow rate of 300 nL/min was performed on an Eksigent NanoLC Ultra 2D Plus connected directly to the LTQ Orbitrap Velos as described previously [22]. Detection parameters on the mass spectrometer were set to maximize the number of precursor ions (peptides) investigated. The instrument was operated in top 20 mode with dynamic exclusion Repeat Count set to 1, Repeat Duration to 90 s, Exclusion List Size to 500, and Exclusion Duration to 60 s.

In vitro T cell activation

Human CD4+T cells were purified from peripheral blood lymphocytes using RosetteSep (STEMCELL Technologies, Vancouver, BC, Canada) and resuspended to 1×10^6 cells/ml in RPMI, 10% fetal calf serum. Human T cell CD3/CD28 expander beads (Invitrogen cat. #111–31D)) were added to purified CD4 + T cells at a ratio of 3 cells:1 bead. Additionally, 10 ng/ml IL-23, 1 ng/ml TGFB, 20 ng/ml IL-6, 10 ng/ml TNFα, 10 ng/ml IL-1β, 10 μg/ml anti-IL-4 and 1 μg/ml anti-IFN-γ were added to the culture. Supernatants were harvested at 5 days and homodimeric and heterodimeric IL-17A and IL-17F were assayed by ELISA.

Immunoprecipitation of IL-17A from Activated CD4+ T Cells

80 ml of activated T cell medium was thawed overnight at 4 °C. The thawed medium was mixed with 15 μg/ml of murine anti-huIL-17A-02 (Pfizer) monoclonal antibody for 2 h at 4 °C under gentle rotation. The complexes were captured with 1 ml of hydrated Protein A-Sepharose (GE Healthcare, Cat#17–0780-01) overnight at 4 °C under gentle rotation. The protein A beads were then sequentially washed with PBS + 1% Tween 20, PBS + 0.1% Tween 20, and PBS + 0.05% Tween 20. The immunoprecipitated pellets were resuspended in non-reducing Tricine sample buffer and loaded onto a 10% Tricine gel for mass-spectrometry analysis.