Proteomic and Glycoproteomic Techniques Used to Investigate PTM Differences in Human Recombinant Proteins Expressed in Different Cell Lines

Recombinant human proteins with human post-translational modifications

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Introduction

Most proteins undergo Post Translational Modification (PTM), which can alter their physical and chemical properties (e.g. MW, pI, folding, stability, activity, immunogenicity, and function). The presence or absence of PTMs may be significant to both the activity and longevity of the protein in a biological system.

Recombinant proteins are dependent on the machinery of the cell line in which they are made to determine their PTMs. Hence the PTMs of human proteins made recombinantly in a human cell line may differ significantly from the same protein made in NSO, CHO, *E.coli* or any other non-human cell line. For example *E.coli* does not possess the type of cellular machinery used for glycosylation in higher organisms, hence proteins produced in an *E.coli* cell line are non-glycosylated. Consequently, the function of this protein may vary significantly from the glycosylated version.

TNFRII is a type 1 transmembrane glycoprotein belonging to the TNF receptor superfamily and has increasingly been recognised as playing an important independent signalling role in chronic inflammatory conditions. Several inflammatory diseases and cancers show unregulated levels of soluble TNFRII or are associated with TNFRII polymorphisms, implicating an important role for TNFRII as a therapeutic target. TNFRII is currently being used to treat moderate to severe rheumatoid arthritis (RA), by binding to TNFα, a pro-inflammatory cytokine, and blocking its interactions with receptors.

Apollo Cytokine Research produce TNFRII-Fc Chimera^{hex} expressed in human cells, as opposed to other commercially available forms TNFRII-Fc, which are produced in CHO or other non human cell lines. The Apollo TNFRII-Fc^{hex} is derived from a DNA sequence encoding the signal peptide and extracellular domain of human TNF Receptor II (TNFRII) (aa1-253) fused to the Fc region of human IgG1 (aa 93-330), and is expressed in modified human 293 cells.

In this study various proteomic and glyco-proteomic methods were used to determine the differences in PTM, in particular glycosylation, that occur on TNFRII-Fc produced recombinantly in human (293 cells) versus non-human (CHO) cells. These methods determine not only the differences in glycosylation but may also give some insight into the possible differences in function of the protein. One-dimensional electrophoresis combined with enzymatic de-glycosylation was used to determine the relative mass of the glycosylated verses the de-glycosylated protein. Enzymatic and chemical de-glycosylation methods combined with MALDI-MS and LC-MS were used to determine the glycosylation sites as well as the N- and O-linked oligosaccharide structures present on the individual proteins.

Methods

Protein de-glycosylation [deN & deO] and 1D SDS PAGE – Proteins were solubilised in 1% ammonium bicarbonate and divided into three aliquots each containing 3-5 µg of protein. The following enzymes were added to the different aliquots and the samples incubated at 37ºC for 12 hr.

Aliquot	Enzymes
1	None
2	PNGase F
3	PNGase F, O-Glycanase, Sialidase A, β(1-4) Galactosidase, β-N-acetylhexosaminidase

An equal volume of 2 × one dimensional sample buffer, and 10 mM DTT was added to each, and then boiled for 5 min. The samples were allowed to cool, before being run on a 4-20% linear gradient Tris-HCl gel. The gels were then stained using a Coomassie blue protein stain (Sigma).

2D-PAGE – Approximately 40 µg of protein was buffer exchanged or precipitated out of the storage buffer, and into an appropriate amount of 2D sample buffer. The protein was then reduced and alkylated using TBP and acrylamide and then used to re-hydrate an 11 cm 3-10 IPG strip. The strip was focussed in the first dimension for approximately 15 hr, until it had reached > 35000 Vhrs. The strip was then equilibrated and the second dimension run on a 4-20% Tris/HCl Criterion gel. Proteins were stained with a fluorescent protein stain.

Release of N- and O-linked oligosaccharides and MS analysis – Proteins cut from PVDF membrane (either electro-blotted following 1D or 2D-PAGE, or dot-blotted) were placed in eppendorf tubes. Methanol was added to wet the membrane, followed by water washes to remove any residual methanol.

N-linked oligosaccharides were enzymatically removed by incubation of the sample at 37ºC for 12 hr with 20 µl of PNGase F (0.125 U/µl). The samples were sonicated for 5 min then the solution (containing the released glycans) was removed from the membrane and placed in a separate tube. The membrane was then washed with 20-30 µl of water, which was removed and added to the same tube. The sample was dried under vacuum. The release N-linked oligosaccharides were then reduced by the addition of 0.5 M sodium borohydride in 10 mM KOH and incubation for 12 hr at 50ºC. The samples were neutralised with glacial acetic acid prior to being cleaned up using cation exchange resin, and borate removal (Schulz et al., Anal. Chem. 2002, 74). The samples were then dried under vacuum and any residual borate removed by repeated addition of 1% acetic acid in 50% methanol and vacuum drying the sample.

O-linked oligosaccharides were chemically released from the PVDF membrane following the N-linked release by incubation of the samples at 50ºC for 12 hr with 50 µl of 0.5 M sodium borohydride in 10 mM KOH. Samples were cleaned up as described for the reduced N-linked oligosaccharides.

The released N- and O-linked oligosaccharides were applied to a Hypercarb porous graphitized carbon column (5 µm Hypercarb, 0.32 × 150 mm, Thermo Hypersil, Runcorn UK) and separated using a linear water-acetonitrile gradient (Wilson et al., J Proteomic Res. 2002, 1, 6) prior to MS and MSn analysis using ESI-MS (Agilent XCT Ultra) in negative mode.

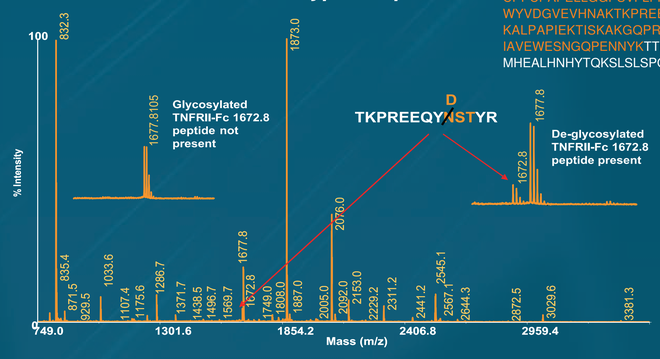
Tryptic digestion and MALDI-MS – Glycosylated and de-glycosylated protein were cut from 1D-PAGE gel. The protein was de-stained, and the gel dried. Trypsin (20 mg/ml in 25 mM NH_4HCO_3, Promega) was added to the gel pieces and the samples incubated for 12 hr at 37ºC. The samples were cleaned up using µC8 ZipTip columns and peptides were eluted and directly spotted onto a MALDI plate using 1 µl of a matrix (3 mg/mL α-cyano-4-hydroxycinnamic acid in 70% (v/v) acetonitrile, 0.01% (v/v) TFA). MALDI-MS was performed on a TOF/TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyser) in positive reflectron mode with automated MSMS performed on the most intense peaks. Spectra were analysed using both Mascot (Matrix science) and FindMod (SwissProt).

TNFRII-Fc^{hex} Sequence Coverage

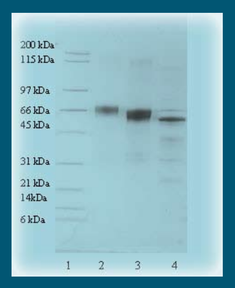
LPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTS
DTVCDSCEDSTYTQLWNWVPECLSCGSRCSSDQVETQACTREQNRICTC
RPGWYCALSKQEGCRLCAPLRKCRPGFGVARPGTETSDVVCKPCAPGTF
SNTTSSTDICRPHQICNVVAIPGNASMDAVCTSTSPTRSMAPGAVHLPQPV
STRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGIPKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQY**DST**YRVVSVLTVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCV
MHEALHNHYTQKSLSLSPGK

Yellow = Sequence Covered
Orange = De-glycosylated peptide only seen after PNGase F treatment
Orange underlined = De-glycosylated N-linked site
Green = unseen glycosylation sites.

MALDI-MS of TNFRII-Fc^{hex} Tryptic Peptides



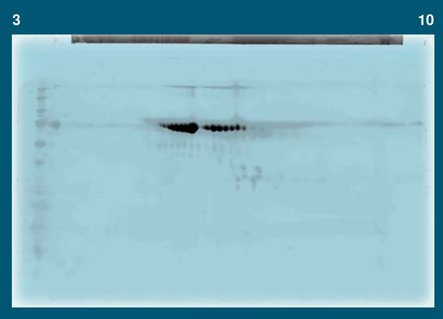
TNFRII-Fc 1D-PAGE [DeN & DeO]



TNFRII-Fc^{hex} (Apollo Cytokine Research) glycosylated and de-glycosylated protein run on 4-20% Tris/HCl gel.

1 = BioRad SDS Mw broad range standards
2 = TNFRII-Fc^{hex}
3 = TNFRII-Fc^{hex} deN-glycosylated
4 = TNFRII-Fc^{hex} deN & O-glycosylated

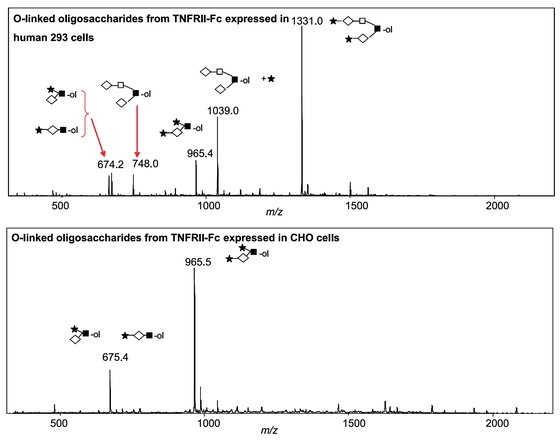
TNFRII-Fc 2D-PAGE



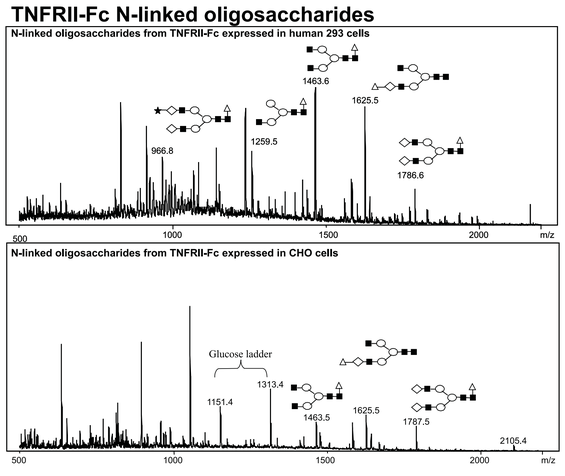
TNFRII-Fc^{hex} (Apollo Cytokine Research) expressed in modified human 293 cells

TNFRII-Fc expressed in CHO cells

TNFRII-Fc O-linked oligosaccharides



TNFRII-Fc N-linked oligosaccharides



Results and Discussion

Protein de-glycosylation [deN & deO] – The 1D-PAGE of the de-N-glycosylated and the de-O-glycosylated TNFRII-Fc^{hex} expressed in modified human 293 cells shows a clear drop in the molecular weight of the protein. The glycosylated protein appears on the 1D-gel as a broad band with a molecular weight of 66 kDa. By removing the N-linked oligosaccharides the protein remains a broad band but drops to a molecular weight of 63 kDa. A further decrease in molecular weight and an increase in resolution occur with the further removal of the O-linked oligosaccharides with TNFRII-Fc^{hex} appearing as a tight band at 58 kDa. These results indicate that the TNFRII-Fc^{hex} is glycosylated and contains both N- and O-linked oligosaccharides.

TNFRII-Fc tryptic peptide analysis – The amino acid sequence of TNFRII-Fc indicates that the protein has 3 potential N-linked sites 2 in the TNFRII region and 1 in the Fc region of the protein. The peptide that contains the theoretical N-linked site N321 is not visible in the spectra of the glycosylated protein, suggesting it may be glycosylated. Confirmation that N321 is glycosylated is provided by observing an additional mass corresponding to the theoretical tryptic peptide +1Da in the spectra of the PNGase F treated TNFRII-Fc sample. The mass is 1Da more than the theoretical peptide due to the conversion of the glycosylated asparagine residue to aspartic acid when de-glycosylated by PNGase F.

The two other potential sites (N149, and N171) occur in a tryptic peptide too large to be seen by MALDI-MS. Treatment with a different enzyme such as Chymotrypsin should result in an amino acid cleavage which will give a peptide capable of being analysed using MALDI-MS.

2D-PAGE of TNFRII-Fc expressed in human 293 cells vs. CHO – There was a significant difference in the 2D-image of TNFRII-Fc protein expressed in human compared to CHO cells. The following table gives a summary of the results.

Source	pI	Molecular weight kDa	No. of isoforms
Human cells (Experimental)	4.6 – 7.05	66	18
CHO cells (Experimental)	5.1 – 6.7	66	26

The difference between the pI range of the TNFRII-Fc expressed in human v's CHO cells suggests a difference in post translational modifications. In particular sialylation is known to increase the acidity of the protein and hence lower the pI.

Oligosaccharide Analysis – The N-linked and O-linked oligosaccharide analysis indicates that while there are several common structures between the human and CHO expressed protein, there were also significant differences that could be the cause of the difference in the number of protein isoforms and their pI seen on the 2D gels. The human expressed TNFRII-Fc contains more complex sialylated as well as neutral O-linked oligosaccharides structures than those found in the CHO expressed protein.

Summary

This work shows that the differences in glycoproteins expressed in different cells can be investigated using proteomic and glycoproteomic techniques. Traditional glycoprotein analytical methods require relatively large amounts of protein whereas the methods used in this study consume minimal quantities of sample.

The data from these preliminary studies shows that Apollo Cytokine Research TNFRII-Fc expressed in human cells has different glycan structures from TNFRII-Fc expressed in CHO cells. These differences may impact the biological activity, and specifically the immunogenicity of the protein.

Further studies are continuing to look at how these differences in glycosylation affect the biological activity of the protein. By combining glycan structural analysis with biological function a better understanding of the importance of cell specific expression in relation to recombinant proteins can be achieved.