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Clarification of the role of *N*-glycans on the common β-subunit of the human IL-3, IL-5 and GM-CSF receptors and the murine IL-3 β-receptor in ligand-binding and receptor activation

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5 are related cytokines that play key roles in regulating the differentiation, proliferation, survival and activation of myeloid blood cells. The cell surface receptors for these cytokines are composed of cytokine-specific α-subunits and a common β-receptor (βc), a shared subunit that is essential for receptor signaling in response to GM-CSF, IL-3 and IL-5. Previous studies have reached conflicting conclusions as to whether *N*-glycosylation of the βc-subunit is necessary for functional GM-CSF, IL-3 and IL-5 receptors. We sought to clarify whether βc *N*-glycosylation plays a role in receptor function, since all structural studies of human βc to date have utilized recombinant protein lacking *N*-glycosylation at Asn^{328}. Here, by eliminating individual *N*-glycans in human βc and the related murine homolog, $\beta_{IL\text{-}3}$, we demonstrate unequivocally that ligand-binding and receptor activation are not critically dependent on individual *N*-glycosylation sites within the β-subunit although the data do not preclude the possibility that *N*-glycans may exert some sort of fine control. These studies support the biological relevance of the X-ray crystal structures of the human βc domain 4 and the complete ectodomain, both of which lack *N*-glycosylation at Asn^{328}.

Keywords: N-linked glycosylation; Interleukin; Cytokine receptor; βc; βIL-3

1. Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3)[2] and interleukin-5 (IL-5) are related cytokines that regulate the differentiation, proliferation, survival and activation of cells of hematopoietic lineages (reviewed in [1]). These cytokines are produced by activated T-cells during immune responses and play an important role in the pathogenesis of many allergic disorders and inflammatory diseases, including asthma, arthritis and multiple sclerosis [2–6]. The effects of GM-CSF, IL-3 and IL-5 on target cells are mediated by receptors composed of cytokine-specific α-subunits and a common β-subunit (βc) [7–13]. Cognate α-subunits bind GM-CSF, IL-3 or IL-5 with low affinities (nanomolar) but in the presence of the β-receptor, high-affinity (picomolar) binding can be

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[2] *Abbreviations used:* IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; hIL-3, human IL-3; mIL-3, murine IL-3; hGM-CSF, human GM-CSF; hIL-5, human IL-5; βc, common β-subunit of the IL-3, IL-5 and GM-CSF receptors; $\beta_{IL\text{-}3}$, mIL-3-specific β-subunit; IL-3α, α-subunit of IL-3 receptor; IL-5α, α-subunit of IL-5 receptor; GM-CSFα, α-subunit of GM-CSF receptor; JAK, Janus kinase; K_d, dissociation constant; ED_{50}, effective cytokine dose for 50% maximal growth response; NAG, *N*-acetyl-glucosamine.

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detected (reviewed in [14]). The formation of a high-affinity β:α:cytokine complex is necessary for the cytoplasmic portions of the receptor subunits to be brought into proximity in the correct orientation to initiate intracellular signaling [15], commencing with the transactivation of Janus kinases, JAK1 and JAK2, which are constitutively associated with the cytoplasmic regions of the α- and β-subunits [16–18].

Insights into the molecular basis for cytokine recognition by the βc-subunit have been provided by the X-ray crystal structure of the complete ectodomain of the human βc-subunit (Fig. 1) [19,20]. Crystals were prepared using recombinant protein expressed from insect cells [19,21] and the elimination of *N*-glycosylation at Asn^{328} by Gln substitution proved essential for the preparation of crystals that diffracted to a suitable resolution (⩽3 Å) to enable structural model building. Prior structures of human βc domain 4 were determined using recombinant protein purified from *Escherichia coli*, a host that does not perform *N*-glycosylation [22,23]. The elimination of N-linked glycosylation is a frequently used strategy to generate crystals that diffract to a higher resolution for structural studies of hematopoietin receptors (for example, ref. [24–28]). However, this approach raises the possibility that the solved structure may be biologically inactive, as the removed oligosaccharide chain may play a role in protein folding, structural stabilization, secretion or cell membrane expression, protection from proteolysis or in mediating macromolecular interactions, such as ligand-binding (reviewed in [29,30]).

To date, studies of the human GM-CSF (hGM-CSF) receptor have reached conflicting conclusions over the involvement of N-linked oligosaccharides in hGM-CSF binding. The tunicamycin-mediated inhibition of N-linked glycosylation in TF-1, a cell line that endogenously expresses the hGM-CSF receptor, permitted near wild-type high-affinity hGM-CSF binding albeit via fewer receptor sites/cell [31]. In contrast, Ding et al. [32] reported that tunicamycin treatment of COS cells transfected with GM-CSFα alone or in combination with the βc-subunit abrogated detectable GM-CSF binding. Subsequently, the elimination of each of the candidate human βc targets for *N*-glycan attachment by Asn to Ala or Asp substitution was reported to completely abolish hGM-CSF high-affinity binding when these mutant βc-subunits were co-expressed with the hGM-CSFα-subunit in COS cells [33]. As a result of the conflicting outcomes of these studies performed using TF-1 and COS cells, it remains unclear whether *N*-glycosylation of the human βc-subunit plays a critical role in the biological function of the GM-CSF, IL-3 and IL-5 receptors, and whether eliminating *N*-glycosylation sites to facilitate structure determination may have resulted in a receptor which is not representative of a biologically active form.

In the present work, we have eliminated individual *N*-glycosylation sites from human βc and an interesting murine homolog, $\beta_{IL\text{-}3}$, using Asn → Gln site-directed mutagenesis and demonstrate using cell-based assays that *N*-glycosylation is critical for neither ligand-binding nor receptor activation. These results provide strong support for the biological relevance of the human βc domain 4 structures [22,23] and the complete ectodomain of human βc [19,20], all of which lack *N*-glycosylation at Asn^{328}.

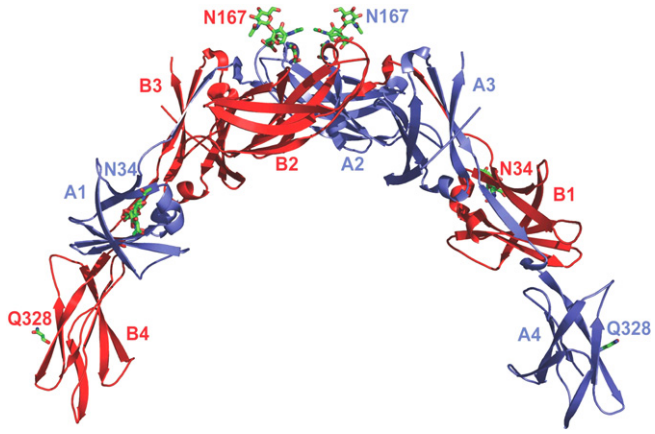

Fig. 1. Structure of the N328Q human βc-subunit ectodomain illustrating sites of *N*-glycosylation. Human βc-subunit ectodomain homodimer (drawn from 1GH7.pdb using PyMol [www.pymol.org]), with the A and B chains colored blue and red, respectively. The fibronectin-III domains that compose the A and B chains are labeled in blue and red text, respectively. N-linked glycans and the Q328 side chain are drawn in sticks colored by atom type (carbon, green; nitrogen, blue; oxygen, red). Q328 was modeled in this cartoon, as no side chain density was observed in the βc crystal structure. The carbohydrates linked to N34 are two *N*-acetylglucosamine (NAG) residues and a mannose; N167 is linked to two NAG residues, with a fucose branched from the first NAG.

2. Materials and methods

2.1. Site-directed mutagenesis of human βc and murine $\beta_{IL\text{-}3}$

The cDNAs encoding the human βc and murine $\beta_{IL\text{-}3}$ receptors were cloned from HL-60 and FDC-P1 total RNA, respectively, as described [34,35]. Site-directed mutagenesis was performed using the QuikChange method (Stratagene) according to manufacturer's instructions and the complete sequences of mutants were verified.

2.2. Expression constructs

For expression in COS7 cells, the cDNAs encoding the hGM-CSFα, mIL-3α, the wild-type or mutant human βc or murine $\beta_{IL\text{-}3}$ subunits were subcloned into the vector pcEX-V3-Xba [34,35]. For expression in CTLL-2 cells, cDNAs encoding the hGM-CSF, hIL-3, hIL-5 or mIL-3α-subunits were subcloned into pEFIRES-N, and cDNAs encoding wild-type or mutant human βc or murine $\beta_{IL\text{-}3}$ subunits were subcloned into pEFIRES-P [36]. Expression constructs were introduced into cells using electroporation [34]. COS7 cells are derived from the kidney of the African green monkey; CTLL-2 cells are derived from mouse T lymphocytes; and the baculovirus expression system utilizes cells derived from the insect, *Spodoptera frugiperda*.