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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}.

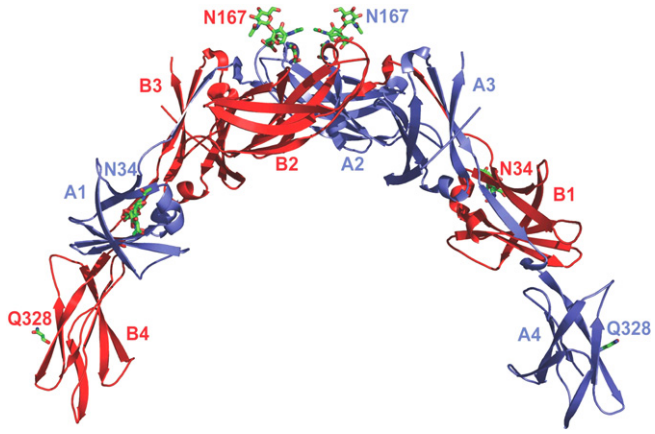

Fig. 1. Structure of the N328Q human βc-subunit ectodomain illustrating sites of *N*-glycosylation. Human βc-subunit ectodomain homodimer (drawn from 1GH7.pdb using PyMol [www.pymol.org]), with the A and B chains colored blue and red, respectively. The fibronectin-III domains that compose the A and B 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*.

It should be noted that mammals make primarily complex and oligomannose *N*-glycans whereas insects make primarily paucimannose and oligomannose *N*-glycans [37].

2.3. Ectodomain and cytokine expression and radiolabelling

Wild-type, N40Q and N328Q $\beta_{IL\text{-}3}$ ectodomains, hGM-CSF, hIL-3, hIL-5, mIL-3 and mIL-2 were produced using the baculovirus expression system [21]. mIL-3 was radiolabelled using the Iodogen method and purified as described [35]. ^{125}I-radiolabelled hGM-CSF was purchased from Perkin-Elmer Life Sciences (Boston, MA) or radiolabelled using the Iodogen method as described for mIL-3 [35].

2.4. Equilibrium binding analysis

A hot saturation assay was used to measure ligand-binding by COS7 cells co-expressing wild-type or mutant β-receptors and relevant α-subunits as described [34]. A cold saturation assay was used to examine direct mIL-3 binding by COS7 cells expressing wild-type or mutant murine $\beta_{IL\text{-}3}$-receptors as before [35].

2.5. Proliferation assays

[^{3}H]Thymidine incorporation assays were performed as described previously [21] to quantify the ability of the wild-type or mutant receptors to deliver a proliferative signal in CTLL-2 stable cell lines.

3. Results and discussion

3.1. The elimination of N-linked glycosylation sites in the human βc and murine $\beta_{IL\text{-}3}$-subunits

Potential N-linked glycosylation sites were identified from the consensus sequence, Asn-X-Ser/Thr (where X is any amino acid except Pro or Asp) [38,39], and eliminated by Asn → Gln mutagenesis. This mutation is conservative and does not normally result in any disruption of protein structure. The sites were mutated individually as receptor misfolding and reduced expression is more likely to occur when several glycosylation sites are eliminated concomitantly (reviewed in [29]). Our studies have shown that all three consensus sites in the human βc ectodomain are utilized for *N*-glycosidic attachment in insect cells. Asn^{34} and Asn^{167} were observed to be glycosylated in crystal structures of the complete ectodomain of human βc (Fig. 1) [19,20], whilst an N328Q mutant βc ectodomain migrated faster than the wild-type analog in SDS–PAGE analysis [21]. Two of these consensus motifs for *N*-glycosylation are conserved in a murine homolog of human βc, the $\beta_{IL\text{-}3}$-subunit, a receptor specific for murine IL-3. We found that both $\beta_{IL\text{-}3}$ sites are subject to *N*-glycosylation, since elimination of glycosylation at Asn^{40} or Asn^{328} by Gln substitution in insect cell-expressed $\beta_{IL\text{-}3}$ ectodomains led to faster migrating bands in SDS–PAGE analysis (data not shown). It is noteworthy that different cell types elaborate different *N*-glycans and that even mammalian cell lines, such as COS7 (simian) and CTLL-2 (murine), may differ from human myeloid cells in glycan chain composition. The studies described herein do not examine whether the origin of the *N*-glycan chain contributes to the specificity of ligand interaction or receptor signaling. Rather, by expressing human βc or murine $\beta_{IL\text{-}3}$ receptors in COS7 and CTLL-2 cells, two cell lines frequently employed for ligand-binding and receptor activation studies, respectively, we have sought to establish the functional consequences of eliminating individual *N*-glycosylation sites to definitively address a long-standing controversy in the field of GM-CSF/IL-3/IL-5 receptor biology.

3.2. N-linked glycosylation sites of human βc are not required for high-affinity hGM-CSF binding

In order to assess the role of human βc *N*-glycosylation in hGM-CSF binding, COS7 cells co-expressing the hGM-CSFα and the wild-type, N34Q, N167Q or N328Q human βc-subunit were used in ^{125}I-hGM-CSF hot saturation binding assays. hGM-CSF binding serves as a useful model system for examining the interaction of the βc-subunit with its ligands, since hGM-CSF high- and low-affinity binding are readily distinguished. This is evident in the curvilinear nature of the Scatchard plot from ^{125}I-hGM-CSF hot saturation binding assays performed on COS7 cells co-expressing the wild-type βc- and hGM-CSFα-subunits (Fig. 2A), where the steepest slope of the curve (on the left) corresponds to high-affinity binding and the shallow slope (on the right of the curve) to low-affinity binding. The presence of two hGM-CSF binding sites on these cells was statistically significant, with the high-affinity site corresponding to hGM-CSF binding in the α:βc complex (K_d, 275 pM) and low-affinity binding corresponding to hGM-CSF binding by GM-CSFα alone (K_d, 18.2 nM) (Table 1 and Fig. 2), as defined in previous studies [34,35]. These K_d values are within the range observed in previous studies of hGM-CSF binding to the wild-type hGM-CSF receptor [33,41]. Comparable K_d values were measured for the N167Q and N328Q mutant βc-subunits (Table 1) indicating that these mutant βc-subunits are correctly folded, transported to the cell surface, and that the oligosaccharide chains attached to Asn^{167} and Asn^{328} in wild-type βc do not play a critical role in the formation of the high-affinity hGM-CSF complex. However, we cannot preclude the possibility that glycans at N167 and N328 of βc play a subtle role in ligand-binding and may be required to exert fine control.

COS7 cells co-expressing N34Q human βc- and hGM-CSFα-subunits showed distinct properties compared to their wild-type, N167Q and N328Q βc-containing counterparts (Table 1 and Fig. 2). Firstly, only about 5% of the number of high-affinity hGM-CSF binding sites were detected on COS7 cells co-expressing the hGM-CSFα and N34Q mutant βc-subunits in hot ligand-binding assays

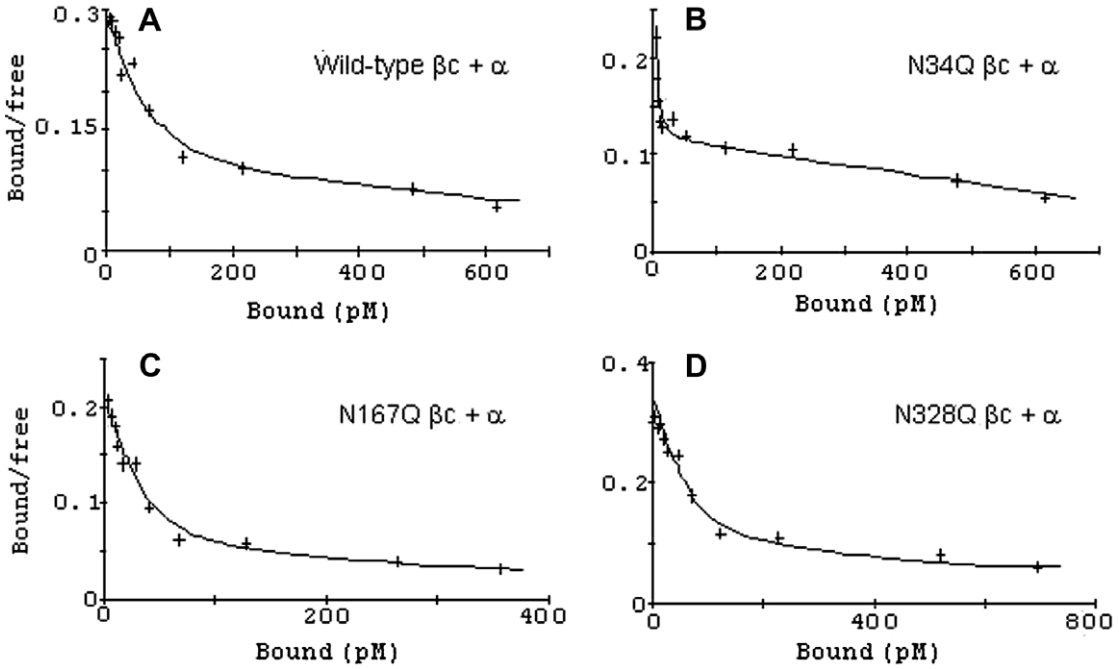

Fig. 2. Human βc-subunit glycosylation is not required for high-affinity hGM-CSF binding. Scatchard plots depicting hGM-CSF binding by COS7 cells co-expressing hGM-CSFα and wild-type (A), N34Q (B), N167Q (C) and N328Q (D) human βc-subunits. Each Scatchard plot shows a representative binding experiment with fitted curves computed by co-analysis of multiple experiments using the program Ligand [40]. The K_d and number of receptor sites/cell determined from ligand co-analysis are reported in Table 1.

compared to the equivalent cells expressing wild-type, N167Q or N328Q βc (Table 1). This finding suggests that a proportion of N34Q βc-subunits may be subject to degradation or defective transportation to the cell surface, resulting in fewer competent hGM-CSF binding receptors on the cell surface. However, even though fewer N34Q βc-containing hGM-CSF receptors were detected on the cell surface compared to their wild-type βc counterparts, it is evident that N34Q βc-subunits on the cell surface are capable of high-affinity hGM-CSF binding. In fact, cells co-expressing N34Q βc and hGM-CSFα exhibited a ~20-fold increase in high-affinity binding compared to their wild-type βc equivalents (Table 1). This increased affinity was observed in three separate binding assays that were performed using cells derived from three separate transfections. The presence of fewer N34Q βc-subunits on the cell surface which are capable of binding hGM-CSF with high-affinity is unlikely to account for the apparent lower K_d for ligand-binding (compare [42]). N34 is adjacent to the domain 1 D–E loop, a loop previously shown to form a disulfide cross-link with the α-subunits in the high-affinity complexes [43,44]. Thus, it is likely that the elimination of *N*-glycosylation from N34 of βc affects the kinetics of ternary complex formation or the relative orientations of the GM-CSFα- and βc-subunits in the high-affinity complex, leading to a higher affinity for GM-CSF.

We next examined the capacity of the triple mutant, N34Q/N167Q/N328Q βc-subunit, devoid of *N*-glycosylation, to bind hGM-CSF when co-expressed in COS7 cells with hGM-CSFα. Owing to very low numbers of high-affinity sites and high associated errors, we had difficulty obtaining an accurate estimate of the K_d. However, the data clearly indicate retention of high-affinity binding in this mutant (Table 1 and Supplementary Figure 1). The low number of detected high-affinity sites is unsurprising, since the N34Q mutation alone reduced the number of high-affinity hGM-CSF binding sites to ~5% of wild-type (described above, this section), and the concomitant elimination of multiple glycosylation sites is known to cause misfolding and/or reduced cell surface expression of other receptors [29]. Our data are consistent with the findings of Shibuya et al. [31], who demonstrated that treatment of TF-1 cells with the *N*-glycosylation inhibitor tunicamycin did not prohibit high-affinity hGM-CSF binding, but did give rise to fewer high-affinity hGM-CSF receptors/cell.

3.3. N-linked glycosylation of the human βc-receptor is not required for activation by human GM-CSF, IL-3 or IL-5

Although the data described in Section 3.2 established that loss of individual N-linked glycosylation sites within the human βc-subunit do not abrogate high-affinity hGM-CSF binding, it was important to examine whether the carbohydrate chains play an important role in receptor activation. Previous studies of the insulin receptor suggest that *N*-glycans play a pivotal role in orientating receptor subunits for receptor signaling [45]. Additionally, the human βc mutants, C62A and C67A, are capable of high-affinity ligand-binding, but exhibit defective receptor activation [44]. Consequently, we established factor-depen-

Table 1
Dissociation constants for cytokine binding by wild-type or glycosylation-deficient receptors expressed in COS7 cells

β-subunit	α-subunit	Cytokine binding assay	Number of sites[a]	High-affinity $K_d \pm$ SE (pM)[b]	High-affinity sites/cell ± SE	Low-affinity $K_d \pm$ SE (nM)	Low-affinity sites/cell ± SE	Number of expts
Wild-type human βc	hGM-CSFα	Hot saturation hGM-CSF	2 $P < 0.001$	275.3 ± 81.8	6822 ± 2137	18.2 ± 0.7	196,695 ± 50,944	2
N34Q human βc	hGM-CSFα	Hot saturation hGM-CSF	2 $P < 0.001$	8.8 ± 5.4	369 ± 67	11.3 ± 1.0	154,829 ± 10,488	3
N167Q human βc	hGM-CSFα	Hot saturation hGM-CSF	2 $P < 0.001$	217.4 ± 36.6	6721 ± 1092	18.8 ± 5.8	156,576 ± 31,796	3
N328Q human βc	hGM-CSFα	Hot saturation hGM-CSF	2 $P < 0.001$	238.8 ± 43.2	8578 ± 1529	25.8 ± 10.0	274,706 ± 77,899	3
N34Q/N167Q/ N328Q human βc	hGM-CSFα	Hot saturation hGM-CSF	2 $P < 0.005$	82.3 ± 104.3	265 ± 210	11.7 ± 1.4	54,274 ± 3425	2
Wild-type murine β_{IL-3}	None	Cold competition mIL-3	1	—	—	18.4 ± 1.0	141,761 ± 9484	4
N40Q murine β_{IL-3}	None	Cold competition mIL-3	1	—	—	21.9 ± 1.1	222,518 ± 8522	2
N328Q murine β_{IL-3}	None	Cold competition mIL-3	1	—	—	23.9 ± 1.4	250,798 ± 11,110	2
Wild-type murine β_{IL-3}	mIL-3α	Hot saturation mIL-3	2 $P < 0.001$	208.5 ± 11.2	18,588 ± 827	45[c]	93,223 ± 10,273	2
Wild-type murine β_{IL-3}	mIL-3α	Hot saturation mIL-3	1[d]	265.5 ± 22.0	10,469 ± 736	—	—	4
N40Q murine β_{IL-3}	mIL-3α	Hot saturation mIL-3	1	273.2 ± 17.2	19,948 ± 910	—	—	2
N328Q murine β_{IL-3}	mIL-3α	Hot saturation mIL-3	1	220.4 ± 13.6	19,347 ± 853	—	—	2

[a] *P*-values were calculated in ligand [40] Statistical significance ($P < 0.05$) indicates that a two-site model better describes the data than a one-site model.

[b] The dissociation constants and number of receptor sites/cell ± the standard errors (SE) were determined from the co-analysis of data obtained from the separate binding experiments (expts) in ligand.

[c] When a two-site binding model was significantly significant in β_{IL-3} hot saturation assays, the low-affinity K_d was fixed as 45 nM to obtain a more accurate estimate of the K_d for high-affinity binding as before [35].

[d] One-site or two-site binding was detected for the wild-type β_{IL-3} in different experiments depending on the relative levels of expression of β_{IL-3} and the mIL-3α-subunit. Data corresponding to both scenarios are presented for comparison.

dent cell lines stably co-expressing the wild-type, N34Q, N167Q or N328Q human βc-subunit and either the hGM-CSF, hIL-3 or hIL-5 α-subunit to compare their proliferation in response to hGM-CSF, hIL-3 or hIL-5, respectively (Table 2). In all cases, the cell lines containing Asn → Gln mutant βc-subunits exhibited quite similar growth responses to those containing wild-type βc, with only minor variations in the effective cytokine dose required for a half-maximal growth response (ED_{50} values) observed (Table 2). Minor variations in ED_{50} between these cell lines are to be expected, since we have previously established that one can anticipate a variation of about 2-fold between the factor-responsiveness of two analogous, independently derived stable cell lines [34]. Therefore, although we do not see any marked differences between the factor-responsiveness of cell lines containing wild-type and glycan-deficient βc-subunits in the present work, we cannot preclude the possibility *N*-glycosylation subtly influences receptor activation.

Interestingly, despite the N34Q mutant βc-subunit exhibiting a ~20-fold higher affinity for hGM-CSF in binding assays performed using COS7 cells (Section 3.2), we did not observe differences in the growth responses of factor-dependent cell lines containing N34Q βc compared to cells expressing wild-type receptors (Table 2). These data indicate that although there were only ~5% of the number of high-affinity hGM-CSF receptors detected on COS7 cells co-expressing the hGM-CSFα- and N34Q βc-subunits (compared to their wild-type equivalents), sufficient receptors are expressed on the cell surface of CTLL-2 cells to transduce a normal proliferative signal in response to wild-type cytokine doses. The fact that ~20-fold higher affinity of the N34Q βc-containing receptor for hGM-CSF is not manifested in increased ligand-responsiveness in proliferation assays is reflective of the complex, multi-step nature of receptor activation, since previous studies suggest that subsequent to high-affinity ligand-binding, the α- and β-subunits must be cross-linked for signaling to occur [43,44] and thus high-affinity cytokine binding may not directly affect the ED_{50} value. However, whilst the N34Q, N167Q and N328Q mutant βc-subunits exhibit wild-type growth responses to hGM-CSF, hIL-3 and hIL-5 in our receptor reconstitution experiments, we cannot exclude the possibility that subtle differences in ligand-recognition, cell surface distribution, receptor activation and resistance to proteolysis may arise and impact upon signal transduction if these βc glycosylation mutations were to occur in vivo.

Table 2
Growth responses of stable CTLL-2 cell lines co-expressing α and wild-type or glycosylation-deficient β-subunits

β-Subunit	Co-expressed α-subunit[a]	Relative ED_{50} values[b]
Wild-type human βc	hGM-CSFα	1
N34Q human βc	hGM-CSFα	1.45
N167Q human βc	hGM-CSFα	0.72
N328Q human βc	hGM-CSFα	0.90
Wild-type human βc	hIL-5α	1
N34Q human βc	hIL-5α	0.79
N167Q human βc	hIL-5α	0.96
N328Q human βc	hIL-5α	0.52
Wild-type human βc	hIL-3α	1
N34Q human βc	hIL-3α	2.74
N167Q human βc	hIL-3α	0.86
N328Q human βc	hIL-3α	0.52
Wild-type murine β_{IL-3}	mIL-3α	1
N40Q murine β_{IL-3}	mIL-3α	0.77
N328Q murine β_{IL-3}	mIL-3α	3.18

[a] CTLL-2 cells stably expressing α-subunits alone exhibited no detectable response to their cognate cytokines, as described previously [34,35].
[b] Averaged ED_{50} values determined from ⩾2 proliferation assays.

3.4. Individual N-linked glycosylation sites of murine β_{IL-3} are not required for low- or high-affinity mIL-3 binding or receptor activation

To augment the findings of our study of hβc *N*-glycosylation, we extended our study to a homolog of hβc found in mice, the β_{IL-3}-receptor. We are currently determining the crystal structure of a ligand:receptor complex containing the β_{IL-3}-subunit. β_{IL-3} is interesting, as unlike βc, β_{IL-3} is able to bind mIL-3 directly with low-affinity [35,46], but like βc, requires co-expression of a mIL-3α-subunit for the formation of a high-affinity complex and receptor activation [47]. Therefore β_{IL-3} provides an opportunity to study the effect of *N*-glycosylation on the direct interaction with mIL-3. Accordingly, COS7 cells expressing the wild-type, N40Q or N328Q murine β_{IL-3}-subunits were assayed for their ability to directly bind ^{125}I-mIL-3 using the cold saturation binding assay. These assays demonstrated that wild-type, N40Q and N328Q β_{IL-3}-receptors are all capable of binding mIL-3 with comparable affinities (Table 1 and Fig. 3), indicating that *N*-glycosylation is not required for this receptor:ligand interaction. Wild-type, N40Q or N328Q β_{IL-3}- and mIL-3α-subunits were also co-expressed in COS7 cells and subjected to hot saturation binding assays, to determine the effect of *N*-glycosylation on high-affinity mIL-3 binding (Table 1 and Fig. 3). As has been shown for other heterodimeric receptor systems, one-site or two-site binding was detected for the wild-type β_{IL-3} in different experiments (Table 1) depending on the relative levels of expression of β_{IL-3}- and the mIL-3α-subunit. However, the K_d determined for high-affinity binding was comparable in both cases. Both the mutants and wild-type β_{IL-3} showed very similar high-affinity binding indicating that the removal of individual glycosylation chains from β_{IL-3} does not prevent the high-affinity binding of mIL-3 in the presence of the mIL-3α-subunit. Factor-dependent CTLL-2 cell lines co-expressing mIL-3α and wild-type, N40Q or N328Q β_{IL-3}-subunits were established and their proliferation in response to mIL-3 was assessed (Table 2). These wild-type and mutant β_{IL-3} cell lines show quite similar growth responses in the mIL-3 titration, with only

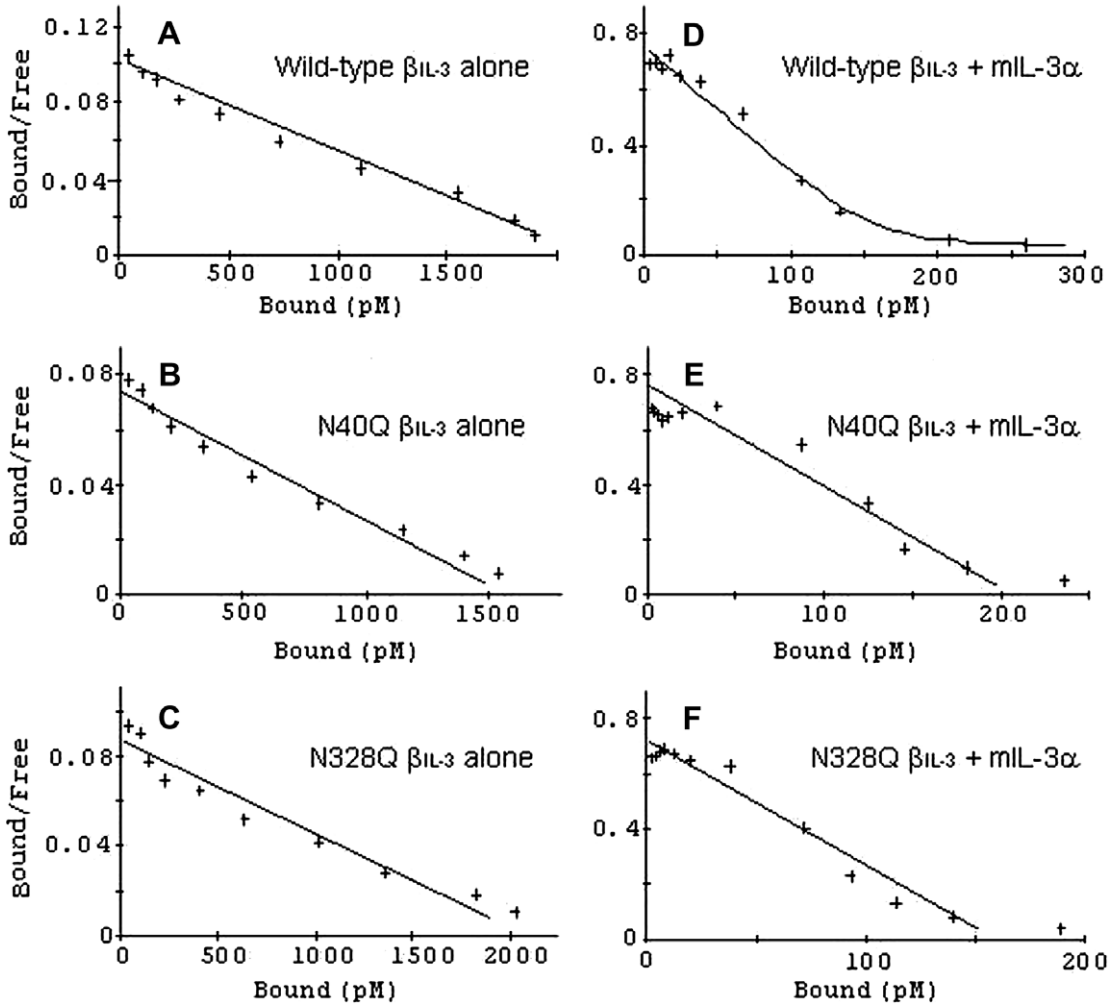

Fig. 3. Murine $\beta_{IL\text{-}3}$-subunit glycosylation is not required for either direct or high-affinity mIL-3 binding. Scatchard plots depicting low-affinity (direct) mIL-3 binding by the wild-type (A), N40Q (B) and N328Q (C) $\beta_{IL\text{-}3}$-subunits in the absence of the mIL-3α-subunit and high-affinity binding by the wild-type (D), N40Q (E) and N328Q (F) $\beta_{IL\text{-}3}$-subunits in the presence of the mIL-3α-subunit. Each Scatchard plot shows a representative binding experiment with fitted curves computed by co-analysis of multiple experiments using the program Ligand [40] yielding statistics reported in Table 1.

minor variations ($\leqslant$3-fold) in ED_{50} values apparent (Table 2), thus providing further support for the notion that *N*-glycosylation does not play a critical role in governing the structure or function of the $\beta_{IL\text{-}3}$-subunit. As noted for our studies of human βc-receptor *N*-glycosylation, we cannot preclude the possibility that subtle effects on ligand-binding, receptor activation, receptor distribution on the cell surface and proteolytic susceptibility, although not detected in the cell-based studies described here, may influence $\beta_{IL\text{-}3}$ signaling should mutations that ablate $\beta_{IL\text{-}3}$ receptor *N*-glycosylation arise in vivo.

4. Conclusion

The results described in the present work demonstrate that the elimination of individual N-linked glycosylation sites does not prevent ligand-binding or receptor activation of the human βc-subunit and a related mIL-3 specific receptor, $\beta_{IL\text{-}3}$. In fact, only the N34Q and N34Q/N167Q/N328Q βc glycosylation site mutants exhibited substantial variance from the wild-type receptor. N34Q βc was found to bind hGM-CSF with ~20-fold higher affinity than the wild-type GM-CSF receptor, albeit via about 5% the number of receptors, yet was capable of delivering a normal proliferative response to GM-CSF, IL-3 and IL-5 when installed in factor-dependent cells. The N34Q/N167Q/N328Q mutant βc exhibited high-affinity hGM-CSF binding but, owing to low numbers of receptors/cell and high associated errors, we were unable to accurately determine the K_d for high-affinity hGM-CSF binding. The low number of detected receptor sites/cell suggests that both the N34Q and N34Q/N167Q/N328Q mutant βc-subunits may exhibit defective transportation to the cell surface or are subject to misfolding or degradation. Interestingly, elimination of the glycosylation site homologous to N34 in human βc in the closely related $\beta_{IL\text{-}3}$ subunit (Asn^{40}) clearly did not result in defective cell surface expression, misfolding or degradation (Section 3.4). In addition, we note that there is no ortholog of human βc N167 present in $\beta_{IL\text{-}3}$, and as such, it is unsurprising that

N167Q human βc exhibited wild-type hGM-CSF binding and proliferative responses to GM-CSF, IL-3 and IL-5 (Sections 3.2 and 3.3).

Overall, our data are in agreement with the results of Shibuya et al. [31] who observed near wild-type high-affinity hGM-CSF binding on TF-1 cells treated with the *N*-glycosylation inhibitor, tunicamycin, with fewer receptor sites/cell detected compared to untreated cells. However, our results completely disagree with the findings of Niu et al. [33] who found that mutagenesis of each of the three potential *N*-glycosylation sites in the βc-subunit abolished high-affinity GM-CSF binding. Although Niu et al. eliminated *N*-glycosylation targets by Asn to Asp or Ala substitutions rather than using our Asn → Gln mutagenesis strategy, we would not expect this factor to influence the findings. Consequently, we have no explanation for the why the βc mutants studied by Niu et al. failed to bind GM-CSF with high-affinity. The present work provides support that the human βc structures which have been determined to date, id est the complete ectodomain [19,20] and domain 4 [22,23] structures, all of which lack glycosylation at Asn^{328}, are biologically relevant conformers of the human βc ectodomain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyto.2008.02.010.

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