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The Role of Interchain Heterodisulfide Formation in Activation of the Human Common β and Mouse β_{IL-3} Receptors*

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Shamaruh Mirza[‡], Jinglong Chen[‡], James M. Murphy[§1], and Ian G. Young[‡2]

From the [‡]Department of Structural Biology, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 0200, Australia and the [§]Division of Molecular Medicine, Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia

The cytokines, interleukin-3 (IL-3), interleukin-5 (IL-5), and granulocyte-macrophage colony-stimulating factor (GM-CSF), exhibit overlapping activities in the regulation of hematopoietic cells. In humans, the common β (βc) receptor is shared by the three cytokines and functions together with cytokine-specific α subunits in signaling. A widely accepted hypothesis is that receptor activation requires heterodisulfide formation between the domain 1 D-E loop disulfide in human βc (hβc) and unidentified cysteine residues in the N-terminal domains of the α receptors. Since the development of this hypothesis, new data have been obtained showing that domain 1 of hβc is part of the cytokine binding epitope of this receptor and that an IL-3Rα isoform lacking the N-terminal Ig-like domain (the "SP2" isoform) is competent for signaling. We therefore investigated whether distortion of the domain 1-domain 4 ligand-binding epitope in hβc and the related mouse receptor, β_{IL-3}, could account for the loss of receptor signaling when the domain 1 D-E loop disulfide is disrupted. Indeed, mutation of the disulfide in hβc led to both a complete loss of high affinity binding with the human IL-3Rα SP2 isoform and of downstream signaling. Mutation of the orthologous residues in the mouse IL-3-specific receptor, β_{IL-3}, not only precluded direct binding of mouse IL-3 but also resulted in complete loss of high affinity binding and signaling with the mouse IL-3Rα SP2 isoform. Our data are most consistent with a role for the domain 1 D-E loop disulfide of hβc and β_{IL-3} in maintaining the precise positions of ligand-binding residues necessary for normal high affinity binding and signaling.

Interleukin (IL)[3]-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are produced by activated T-cells during immune responses and play key roles in the regulation of inducible hematopoiesis and inflammation. Dysregulation of the production and activity of these three cytokines is known to underpin the pathogenesis of many allergic disorders and inflammatory diseases of the lung, such as asthma. IL-5 is the primary regulator of eosinophilic inflammation (1, 2), whereas IL-3 plays a central role in basophil and mast cell responses to parasite infections (3). GM-CSF is thought to be centrally involved in chronic inflammatory diseases, such as arthritis and multiple sclerosis (4). GM-CSF also regulates the differentiation and activation of alveolar macrophages, and mutations affecting GM-CSF receptor α (GM-CSFRα) have been detected in patients with pulmonary alveolar proteinosis (5). Clinically, GM-CSF offers immense potential as an immune stimulant and vaccine adjuvant in cancer patients (4).

Human IL-3, IL-5, and GM-CSF exert their effects on target cells via cell surface receptors composed of cytokine-specific α subunits and a common β subunit (hβc). Each cytokine can bind their cognate α subunits with low affinities (1–50 nM), but in the presence of hβc, high affinity complexes are formed (30–500 pM) (6–9). Notably, the hβc subunit does not detectably bind any of its three cytokine ligands in the absence of the cognate α subunits. The activated receptor complexes formed with each of the three ligands activate Janus kinase 2 (10, 11), leading to the phosphorylation of tyrosine residues located in the βc cytoplasmic domain (12, 13) and the induction of several signaling pathways (14). In mice, in addition to the mouse βc receptor, there is an IL-3-specific β receptor (denoted β_{IL-3}) that binds IL-3 directly but still requires mIL-3Rα for signaling (15). Structurally, the IL-3, IL-5, and GM-CSF receptors belong to the cytokine class I receptor superfamily, which includes receptors for growth hormone, erythropoietin, gp130, and IL-4/IL-13 (16). The unifying structural feature of this family is the cytokine receptor homology module (CRM) present in the receptor ectodomains, a module composed of two fibronectin III domains (reviewed in Ref. 16). X-ray crystal structures of the ectodomains of several members of this receptor family have been solved over the past 2 decades, revealing that the two fibronectin-III domains that compose each CRM are universally in an approximately orthogonal configuration relative to one another, with residues from loops at the receptor "elbow" region serving key functions in ligand binding (17–22).

In 2001, we reported the crystal structure of the complete ectodomain of the hβc subunit (23), which was a radical depar-

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[1] To whom correspondence may be addressed. Tel.: 61-3-93452407; Fax: 61-3-93470852; E-mail: jamesm@wehi.edu.au.

[2] To whom correspondence may be addressed. Tel.: 61-2-61250607; Fax: 61-2-61250415; E-mail: Ian.Young@anu.edu.au.

[3] The abbreviations used are: IL, interleukin; hIL, human IL; mIL, mouse IL; GM-CSF, granulocyte-macrophage colony-stimulating factor; hGM-CSF, human GM-CSF; GM-CSFRα, IL-13Rα1, IL-5Rα, or IL-3Rα, α subunit of the GM-CSF, IL-13, IL-5, or IL-3 receptor, respectively; D1, N-terminal Ig-like domain of IL-13Rα1, GM-CSFRα, IL-5Rα or IL-3Rα; βc, common β subunit of the GM-CSF, IL-3 and IL-5 receptors; hβc, human βc; β_{IL-3}, mouse IL-3-specific β receptor subunit; CRM, cytokine receptor homology module; FACS, fluorescence-activated cell sorting; PE, phycoerythrin.

ture from the growth hormone receptor paradigm because the hβc exists entirely as a preformed, intertwined dimer due to a novel strand swapping between protein chains (Fig. 1*A*). Subsequently, we established that despite the marked deviation from the growth hormone receptor paradigm, a novel elbow region formed between domains 1 and 4, contributed by the two protein chains of the homodimer, served as an unusual ligand-binding elbow region (24, 25). This unusual model for ligand binding was recently validated by the crystal structure of the GM-CSF receptor complex (26). The IL-3Rα, IL-5Rα, and GM-CSFRα subunits are part of a subgroup of the class I cytokine receptor superfamily that possess an additional Ig-like domain (D1) N-terminal to the CRM in their ectodomains. In other cytokine receptor systems, structural studies have clearly established that Ig-like domains play important roles in mediating cytokine binding and receptor activation (*e.g.* in the IL-6·IL-6Rα·gp130 (27) and IL-13Rα1·IL-13 (28) complexes. In the case of IL-5, alanine-scanning mutagenesis of the IL-5Rα subunit identified residues Asp^{55}, Asp^{56}, and Tyr^{57} in IL-5Rα domain D1 as essential determinants of IL-5 binding (29, 30). Additionally, deletion of domain D1 of the hIL-5Rα was shown to block IL-5 signaling (29). By comparison, we have recently shown that a naturally occurring splice variant of IL-3Rα, which lacks domain D1 (designated "SP2"; Fig. 1*B*), is capable of binding IL-3 with high affinity and is competent for signaling (31). Collectively, these studies illustrate that details of the mechanisms underlying activation by IL-3, IL-5, and GM-CSF receptors are still emerging, and recent structural and biochemical studies suggest that receptor activation is likely to differ markedly from the paradigms established for other class I cytokine receptors.

Although it is well established that heterodimerization with the α subunits is required for hβc activation, evidence has also been reported for an additional step in receptor activation. It was shown that a proportion of the heterodimers involving hβc were disulfide-cross-linked (32). A critical link between the disulfide cross-linking and receptor activation was suggested by the properties of hβc mutants in which the D-E loop disulfide had been mutated (33). These mutants retained high affinity binding but lost intersubunit cross-linking and receptor activation (33). The domain 1 D-E loop disulfide is highly conserved in the hβc, mβc, and β_{IL-3} receptors and is additional to the two structural disulfides present in the CRM of these receptors. It was hypothesized that hβc receptor activation requires the initial formation of a high affinity complex with the α subunit and cytokine ligand prior to intersubunit cross-linking between the D-E loop disulfide on hβc and a free SH group on domain 1 of the α receptor (33). Implicit in the hypothesis is that mutation of the D-E loop disulfide does not result in any structural perturbation that could prevent receptor activation. The heterodisulfide hypothesis has been widely accepted and is potentially very important in understanding the activation of the hβc receptor. However, a number of details of the mechanism have not been elucidated. For example, the Cys residues involved in the interchain disulfide in the respective α receptors have not been identified, and it is not clear how the mechanism would operate with a recently characterized splice variant of IL-3Rα (denoted SP2), which is capable of ligand binding and receptor activation despite lacking D1 (31). Furthermore, based on the x-ray crystal structure of the hβc ectodomain (23) and subsequent biochemical studies (24, 25), we identified a previously unrecognized role for domain 1 of hβc in forming the functional epitope for ligand binding and receptor activation (Fig. 1*C*). This novel function for domain 1 of hβc was validated in the recently solved crystal structure of the activated hGM-CSFR complex (26). Intriguingly, there is a notable absence of electron density for domain 1 of the GM-CSFRα subunit and no apparent means by which the remaining domains (domains 2 and 3, the CRM) of the GM-CSFRα could contribute to a disulfide cross-link with the hβc domain 1 D-E loop within the hexameric complex, although a cross-link between adjacent hexamers may still be possible (26).

In the present work, we have sought further details of the heterodisulfide mechanism by studying both the hβc and β_{IL-3} receptors. We have confirmed the original finding that mutation of the D-E loop disulfide in domain 1 of hβc does not destroy high affinity binding for hIL-3 (in the presence of the full-length hIL-3Rα, the "SP1" isoform) or for hGM-CSF in the presence of hGM-CSFRα, although the binding properties are different from those of the wild type receptors. However, when a hIL-3Rα isoform lacking the N-terminal Ig-like domain (the SP2 isoform) was used, mutational disruption of the hβc domain 1 D-E loop disulfide resulted in complete loss of the high affinity binding and signal transduction, in absolute contrast to the high affinity binding and signaling observed in parallel experiments with wild type hβc. Analogous domain 1 D-E loop mutations in the related β_{IL-3} receptor not only led to a loss of high affinity mIL-3 binding and downstream signaling when co-expressed with a mIL-3Rα SP2 ortholog but also ablated the unique capacity of β_{IL-3} to directly bind IL-3. These data are most consistent with a role for the domain 1 D-E loop disulfide within the orthologous receptors, hβc and β_{IL-3}, in maintaining the three-dimensional structure of domain 1 rather than the postulated unique role of this disulfide in heterodisulfide formation. In agreement with this, we found that the only unpaired cysteine residue in the hIL-5Rα ectodomain, Cys^{66} in the Ig-like domain, could be mutated to alanine without compromising the capacity of this α subunit to signal normally when co-expressed with wild type hβc in the presence of hIL-5. These data suggest a revised model for receptor activation in which the integrity of the D-E loop disulfide in domain 1 of hβc and β_{IL-3} is essential for maintaining the precise three-dimensional positions of essential ligand-binding residues that are necessary for high affinity binding and receptor activation. These data do not support a role for heterodisulfide cross-linking between the IL-3, IL-5, and GM-CSF α subunits and the D-E loop disulfide in domain 1 of hβc and β_{IL-3} as a key step in receptor activation.

EXPERIMENTAL PROCEDURES

Cells and DNA Transfections—COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. CTLL2 cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and murine IL-2 at 10 units/ml. Cells were cultured in 5% CO_2. The growth medium for all cell lines was supplemented with 60 μg/ml benzyl penicillin, 100 μg/ml streptomycin, and 10 μg/ml

gentamycin. COS7 cells were harvested using trypsin and electroporated with 10 μg of wild type or mutant mIL-3Rα SP1, mIL-3Rα SP2, hGM-CSFRα, hIL-3Rα SP1 or SP2 and 25 μg of wild type or mutant hβc or β_{IL-3} constructs at 200 V and 960 microfarads. Binding studies and antibody staining were performed on cells 64–68 h after transfection. CTLL2 cell lines stably expressing wild type or mutant hIL-5Rα, mIL-3Rα SP1, mIL-3Rα SP2, hGM-CSFRα, hIL-3Rα SP1, or hIL-3Rα SP2 and wild type or mutant hβc or β_{IL-3} subunits were generated in two steps with DNA introduced by electroporation, essentially as described previously (34).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the QuikChange method with *Pfu* Turbo Ultra DNA polymerase (Stratagene) according to the manufacturer's instructions to prepare mutant hβc, β_{IL-3}, and hIL-5Rα cDNAs from wild type cDNA templates using appropriate mutation-encoding synthetic oligonucleotides. The complete sequences of wild type and mutant cDNAs were verified by Big Dye Terminator cycle sequencing (Applied Biosystems, Gladesville, Australia).

Expression Constructs—For expression in COS7 cells, the cDNAs encoding wild type or mutant hβc, β_{IL-3} mIL-3Rα isoforms, hIL-3Rα isoforms, and hIL-5Rα subunits were subcloned into pCEX-V3-Xba, a vector derived from pCEX-V3 (35). For expression in CTLL2 cells, cDNAs encoding wild type or mutant human hIL-5Rα/mIL-3Rα isoforms/hGM-CSFRα/hIL-3Rα isoforms were subcloned into pEF-IRES-N, and the hβc and β_{IL-3} subunits were subcloned into pEF-IRES-P (36) (from S. Hobbs (Institute of Cancer Research, London, UK)).

Cytokines and Radiolabeling—Human GM-CSF, IL-3, and IL-5 and mouse IL-3 and IL-2 were produced using the baculovirus expression system, as described previously (34). Purified GM-CSF, hIL-3, and mIL-3 were radiolabeled using the Iodogen reagent as described previously (25, 37). Radiolabeled ligands were stored at 4 °C and used for up to 14 days. ^{125}I was purchased from PerkinElmer Life Sciences and stored at room temperature.

Proliferation Assays—[^{3}H]Thymidine incorporation assays were performed as described previously (34) to determine the capacity of the wild type or mutant receptor subunits to deliver a proliferative signal in CTLL2 stable cell lines in response to ligands.

Flow Cytometry—Two steps of flow cytometry were used to detect the heterogeneously expressed wild type or mutant hβc and wild type or mutant β_{IL-3} on the surface of CTLL2 and COS7 cells. Cells were harvested and washed with cold phosphate-buffered saline, and then cells were incubated with a 1:200 dilution of primary antibody in FACS incubation buffer (0.2% bovine serum albumin, 5% fetal bovine serum in cold phosphate-buffered saline) for 1 h. After three washes with FACS washing buffer (0.2% bovine serum albumin in cold phosphate-buffered saline), the cells were then incubated with secondary antibody (1:500 dilution of the relevant isotype of streptavidin-PE or IgM conjugated with PE) for 0.5 h. Cells were washed three times with FACS washing buffer before being subjected to FACS performed on a FACScan flow cytometer (BD Biosciences). The same concentration of the relevant isotypes of immunoglobin of primary antibodies was used as negative control. The primary monoclonal antibodies were 3D7 (biotinylated anti-CDw131, for detection of hβc; BD Pharmingen) and AIC2 (for detection of mouse β_{IL-3}; MBL Medical and Biological Laboratories).

Equilibrium Binding Analysis—Hot saturation binding experiments were performed on COS7 cells transiently transfected with DNA constructs encoding the relevant receptors or using CTLL2 cells stably expressing the relevant receptors, as described previously (24). The dissociation constants (K_d) and B_{max} were determined from specific binding data obtained from hot saturation assays by using the programs EBDA (38) and LIGAND (39), as before (24). In order to assess the capacity of β_{IL-3} subunits to bind mIL-3 directly, cold saturation assays were performed on COS7 cells transfected with cDNAs encoding wild type or mutant β_{IL-3} subunits in the absence of the mIL-3Rα cDNA, as described previously (25). Scatchard plots were used for visual evaluation of the quality of the data and their fits to one site and two-site models.

RESULTS

Cys^{67} Is Essential for Growth Signaling by GM-CSF, IL-3, and IL-5 through hβc—Our x-ray structure of the ectodomain of hβc (23) established that Cys^{62} in the domain 1 D-E loop and Cys^{67} at the beginning of the E strand form a disulfide (referred to as domain 1 D-E loop disulfide; Fig. 1*C*). The D-E loop disulfide is well conserved in hβc, mβc, and β_{IL-3} and is additional to the two structural disulfides present in the CRMs of these receptors (Fig. 1*D*). To disrupt the domain 1 D-E loop disulfide, Cys^{67} of hβc was mutated to alanine. C67Ahβc was installed in parental CTLL2 cell lines stably expressing either IL-3Rα SP1, IL-3Rα SP2, IL-5Rα, or GM-CSFRα. In contrast to wild type hβc, C67Ahβc was completely unable to support growth on hIL-3 with either hIL-3Rα SP1 or SP2. It was also unable to support growth on hIL-5 or hGM-CSF when co-expressed with IL-5Rα or GM-CSFRα, respectively (Fig. 2*A*). This effect was not due to poor expression because the mutant receptor was present at levels comparable with those of the wild type hβc on the surface of CTLL2 cells (Fig. 2*B*). Thus, the hβc domain 1 D-E loop disulfide is critical for growth signaling with all three cytokines through hβc even in the case of hIL-3Rα SP2, a splice variant of hIL-3Rα lacking the N-terminal Ig-like domain.

High Affinity Binding Properties of C67Ahβc—The critical observation that led to the development of the heterodisulfide hypothesis of hβc activation was the retention of high affinity binding with hβc mutants in which the domain 1 D-E loop disulfide was mutated (33). If this disulfide were playing a structural role, its mutation would be expected to cause a loss of high affinity binding as was observed in the original work for mutation of the structural disulfide bond between strands D and E (33). Here, we examined the capacity of the C67A mutant hβc to mediate high affinity binding with hIL-3 in the presence of hIL-3Rα SP1 and with hGM-CSF in the presence of hGM-CSFRα (Fig. 3*A* and Table 1).

We were able to verify the original findings of retention of high affinity binding in both cases. With hIL-3Rα SP1 in CTLL2 cells, wild type hβc gave a K_d of 217 pM. However, although high affinity binding was observed with hIL-3Rα SP1 plus C67Ahβc, it was clearly different from that of the wild type receptor. The binding appeared to be of very high affinity (K_d = 3 pM), and there were few

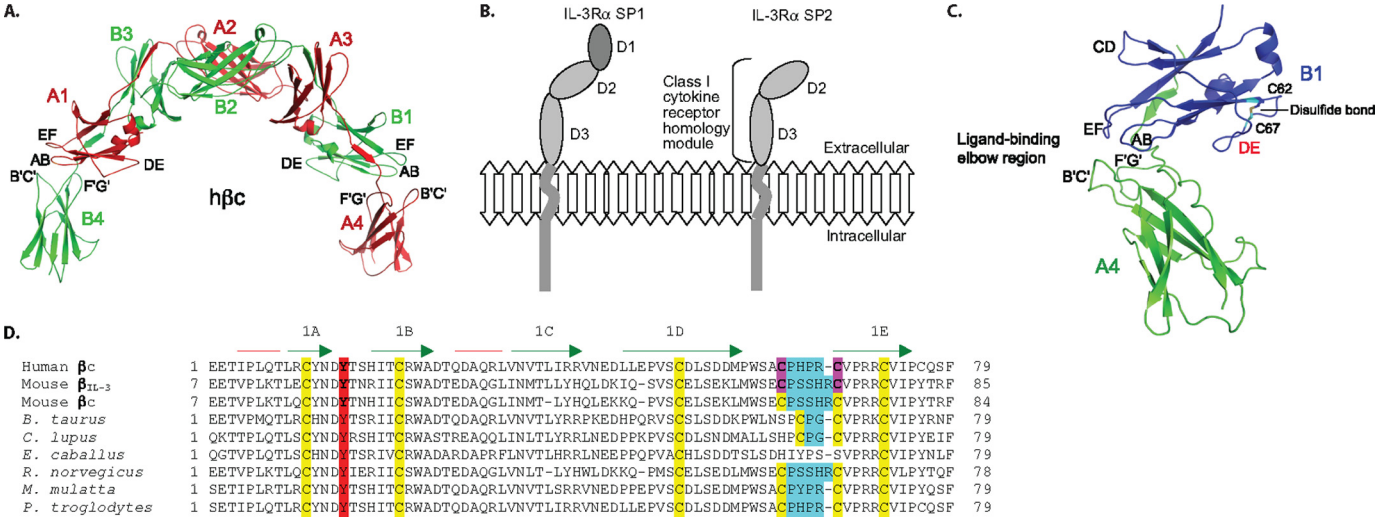

FIGURE 1. *A*, structure of the βc homodimer. Chain A is *green*, and chain B is *red*. Domains of both chain A and B are labeled in *green* and *red text*, respectively. The loops of domain 1 and 4 relevant to ligand-receptor interaction, receptor assembly, and structural integrity are labeled in *black text*. The structure of hβc is taken from Protein Data Bank entry 1gh7. The schematic diagram was drawn using PyMOL (DeLano Scientific LLC). *B*, schematic models of IL-3Rα SP1 and SP2 isoforms showing structural domains. In human and mouse SP2 isoforms, deletions of 234 and 279 bp, respectively, occur in D1. *C*, domain 1-domain 4 elbow region of hβc showing the domain 1 D-E loop. Domain 4 from chain A and domain 1 from chain B have been colored *green* and *blue*, respectively. The Cys^{62}-Cys^{67} disulfide is colored *yellow*, and the D-E loop is labeled in *red text*. The loops of domains 1 and 4 relevant to ligand-receptor interaction are labeled in *black text*. The schematic diagram was drawn using PyMOL. *D*, multispecies alignments of βc covering the region of the domain 1 D-E loop disulfide. Conserved Cys residues are highlighted in *yellow*, and Cys^{62} and Cys^{67} of hβc and Cys^{67} and Cys^{73} of β_{IL-3} are highlighted in *purple* and in *boldface type*. The residues between the conserved cysteines (Cys^{62}–Cys^{67}) composing the disulfide motif are highlighted in *blue*. The critical high affinity ligand binding residues Tyr^{15} and Tyr^{21} of hβc and mβ$_{IL-3}$, respectively, are highlighted in *red* and in *boldface type* and are well conserved among species. The secondary structure features observed in the βc structure are marked *above* the *sequence*; *green arrows* represent β strands, and *red lines* represent helical regions. In the alignment, the WS*X*WS motif and other key ligand binding residues are well conserved. In the figure, only the region of the alignment covering the residues up to 79, 85, and 84 of hβc, β_{IL-3}, and mβc, respectively, is shown.

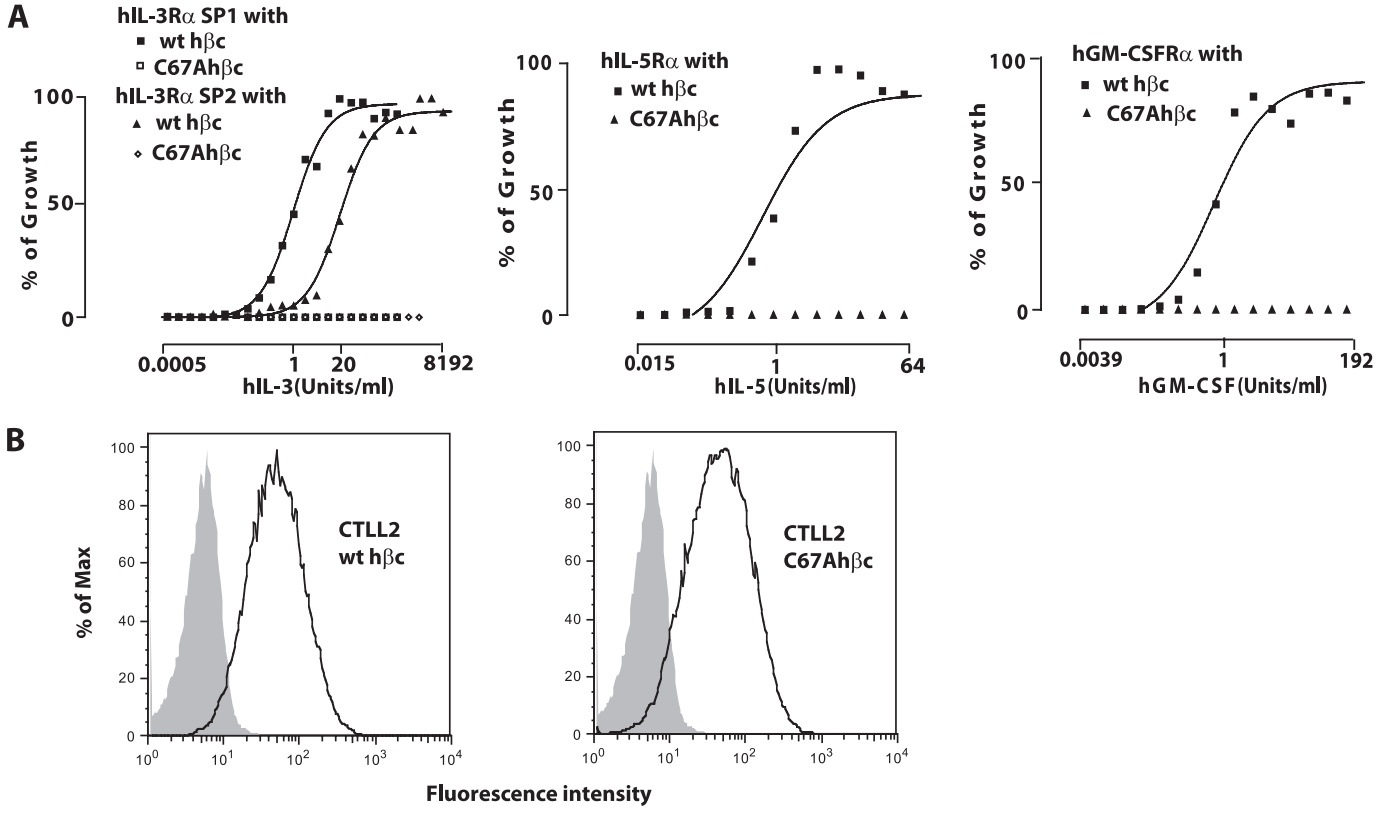

FIGURE 2. **Growth signaling and cell surface expression properties of C67Ahβc receptor in CTLL2 cells.** *A*, plots depict the percentage of growth in CTLL2 cells stably expressing wild type (*wt*) or mutant human receptors in response to a serial dilution of growth factor as labeled in the figure. The data shown are representative of data from at least two experiments. *B*, cell surface expression of wild type hβc or C67Ahβc in stably transfected CTLL2 cells was detected by flow cytometry following the method described under "Experimental Procedures." Cells stained with only the PE-conjugated antibody were used as a control for each assay (depicted by the *gray shaded area*).

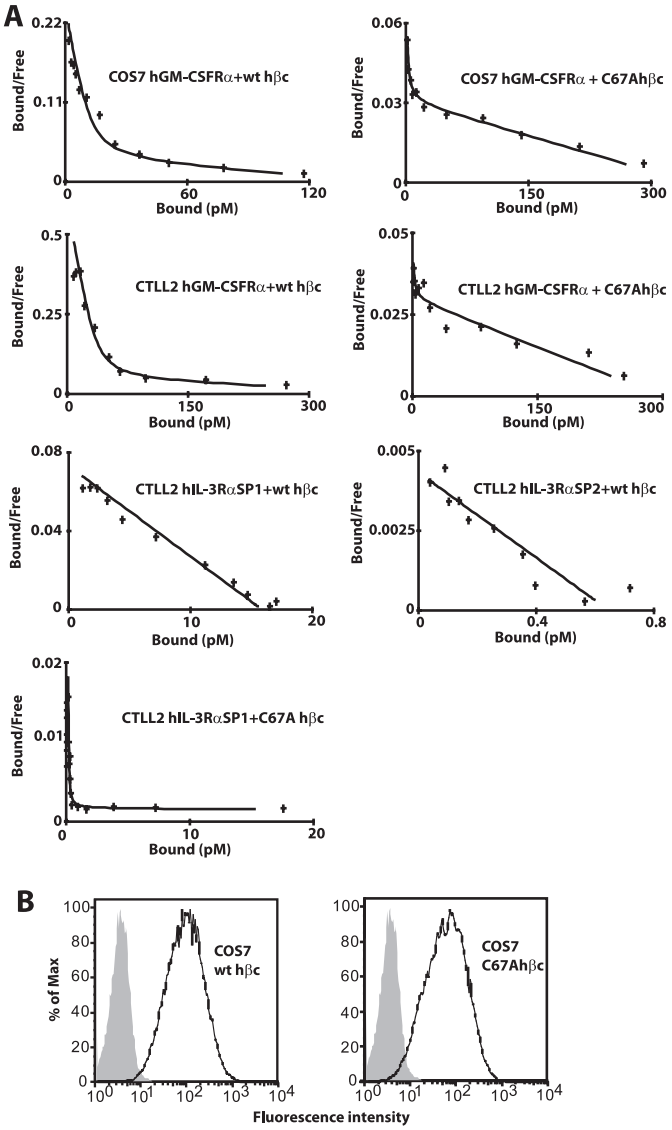

FIGURE 3. **Ligand binding properties of C67Ahβc and its cell surface expression in COS7 cells.** *A*, Scatchard plots of ^{125}I-labeled GM-CSF or hIL-3 hot saturation binding data for COS7 or CTLL2 cells expressing hGM-CSFRα or hIL-3Rα SP1 or hSP2 plus wild type (*wt*) hβc or C67Ahβc. Each plot in the figure is labeled with the cells used followed by the receptors being measured. Data from a representative hot saturation binding experiment are shown in each plot with the line of best fit determined by co-analysis of data from several binding experiments using LIGAND (39). The derived K_d values are shown in Table 1. Curvilinear plots, such as that in the *top left panel*, are indicative of two-site binding models, whereas one-site binding models show a linear fit to the data. *B*, cell surface expression of wild type and mutant hβc in COS7. Cells stained with only the PE-conjugated antibody were used as a control for each assay (depicted by the *gray shaded area*).

high affinity sites relative to the numbers of low affinity sites (which correspond to ligand binding by the α subunit). This was despite comparable expression of the wild type and mutant receptors (see below). High affinity hGM-CSF binding was also examined in both COS7 and CTLL2 cells (Table 1 and Fig. 3*A*). Using cells co-expressing hGM-CSFRα and wild type hβc, K_d values of 63–68 pM were obtained, but once again, the analogous experiment examining the C67Ahβc mutant receptor revealed a higher apparent affinity (K_d ~16–18 pM) via few high affinity binding sites relative to the number of low affinity sites. Reduction in the number of competent receptors and a tendency to higher affinity binding was also observed in the original work with both IL-3 and GM-CSF (33). Extension of the binding studies to hIL-3 in the presence of hIL-3Rα SP2, however, gave a completely different result. There was a complete loss of high affinity binding when the hβc domain 1 D-E loop disulfide was disrupted. With wild type hβc and hIL-3Rα SP2, a K_d of 150 pM was obtained in CTLL2 cells (Table 1 and Fig. 3*A*). In contrast, with hIL-3Rα SP2 plus C67Ahβc, no high affinity binding could be detected. These data indicate a structural role for the domain 1 D-E loop in hβc rather than a role in hβc·α heterodisulfide formation. A loss of structural integrity of C67Ahβc provides an explanation for the abnormal high affinity binding seen with this receptor in combination with hIL-3Rα SP1 and hGM-CSFRα, particularly the low number of binding sites. None of the above effects on binding could be explained by poor expression because the level of C67Ahβc on the cell surface was comparable with that of the wild type hβc in CTLL2 (Fig. 2*B*) and in COS7 cells (Fig. 3*B*). Additionally, flow cytometry demonstrated that cell surface expression of hGM-CSFRα, hIL-3Rα SP1, and hIL-3Rα SP2 were equivalent in both wild type and mutant hβc-expressing CTLL2 cell lines (data not shown), as one would expect, because the hβc subunits were derived from parental CTLL2 cell lines stably expressing the relevant α subunit.

Cys^{73} Is Essential for Growth Signaling by mIL-3 through β_{IL-3}—We also included the β_{IL-3} receptor, which is a closely related mouse ortholog of hβc, in the study. The domain 1 D-E loop disulfide is highly conserved in the hβc, mβc, and β_{IL-3} receptors (Fig. 1*D*). Structural alignment indicated that Cys^{67} and Cys^{73} form the domain 1 D-E loop disulfide in the β_{IL-3} receptor. Because previous work had shown that the phenotype was the same irrespective of which Cys of the disulfide was mutated for hβc (33), we mutated Cys^{73} to alanine in β_{IL-3}. C73Aβ_{IL-3} was stably expressed in CTLL2 cells co-expressing either the SP1 or SP2 isoforms of mIL-3Rα. Wild type β_{IL-3} supported growth with either isoform of mIL-3Rα, with the SP2 isoform requiring higher levels of mIL-3 as described previously (31). In contrast, no growth signaling was detectable with C73Aβ_{IL-3} when co-expressed with either the SP1 or SP2 isoforms of mIL-3Rα (Fig. 4*A*). This was not due to faulty expression because C73Aβ_{IL-3} was expressed on the cell surface at levels similar to that of the wild type β_{IL-3} (Fig. 4*B*). Further, the wild type and C73Aβ_{IL-3} subunits were installed into parental CTLL2 cell lines stably expressing either the SP1 or SP2 mIL-3Rα isoforms, and as one would expect, equivalent levels of mIL-3Rα subunit expression were observed for each of the derived wild type and C73Aβ_{IL-3} cell lines using flow cytometry (SP1) or quantitative reverse transcription-PCR (SP2) (data not shown). Quantitative reverse transcription-PCR was used to verify SP2 mIL-3Rα subunit expression because no anti-SP2 antibody is presently available. Thus, the D-E loop disulfide in domain 1 of β_{IL-3} is critical for growth signaling with mIL-3, even with mIL-3Rα SP2, an α subunit isoform lacking the N-terminal Ig-like domain.

Ligand Binding Characteristics of C73Aβ_{IL-3}—The C73Aβ_{IL-3} mutant was tested for its ability to support high affinity binding in both CTLL2 cells and COS7 cells. High affinity binding with mIL-3Rα SP1 plus wild type β_{IL-3} gave K_d values of ~160–170 pM in either COS7 or CTLL2 cells (Table 2

TABLE 1

Dissociation constants for human GM-CSF or IL-3 binding to cells expressing hGM-CSFRα or hIL-3Rα and wild type or C67Ahβc subunits

Cell line	Human α subunit	hβc	High affinity K_d ± S.E.[a]	High affinity sites/cell ± S.E.	Low affinity K_d ± S.E.	Low affinity sites/cell ± S.E.	No. of experiments
			pM		*nM*		
COS7	hGM-CSFRα	Wild type	68 ± 13.5	1765 ± 245	10[b]	24,884 ± 2211	4
COS7	hGM-CSFRα	C67A	19 ± 9	26 ± 6	10[b]	6987 ± 317	4
CTLL2	hGM-CSFRα	Wild type	63 ± 11.5	6934 ± 811	10[b]	84,890 ± 8270	2
CTLL2	hGM-CSFRα	C67A	17 ± 43	68 ± 48	10[b]	37,520 ± 1375	2
CTLL2	hIL-3RαSP1	Wild type	217 ± 11.7	1910 ± 70	—[c]		2
CTLL2	hIL-3RαSP1	C67A	3.5 ± 2.6	24 ± 2.9	100[d]	19,371 ± 1162	2
CTLL2	hIL-3RαSP2	Wild type	150 ± 14	78 ± 5.2	—[c]		3
CTLL2	hIL-3RαSP2	C67A	ND[e]				2

[a] Binding was determined using the hot saturation binding assay and K_d ± S.E. determined by co-analysis of data from multiple experiments using LIGAND (39).
[b] To improve the accuracy of determination of the high affinity K_d, the low affinity site K_d (for hGM-CSFRα binding) was fixed at 10 nM, a value that is within the range of hGM-CSFRα binding reported previously (1–10 nM) (43–45). Without fixing, the low affinity K_d values ranged from 8 to 16.2 nM.
[c] —, a one site-binding model was statistically significant.
[d] To improve the accuracy of determination of the high affinity K_d, the low affinity site K_d (for hIL-3RαSP1 binding) was fixed at 100 nM, according to established protocols (46).
[e] ND, binding was not detectable above background.

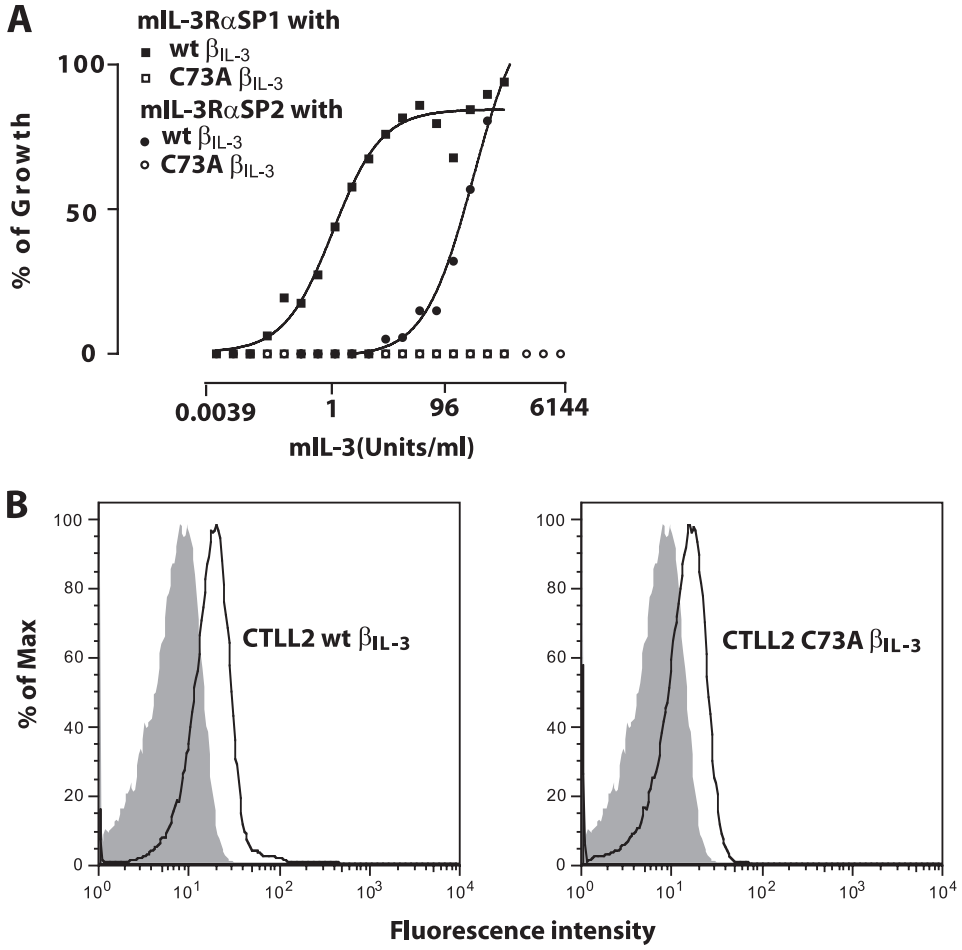

FIGURE 4. **Growth signaling and cell surface expression properties of the C73Aβ_{IL-3} receptor in CTLL2 cells.** *A*, proliferation of CTLL2 cells stably expressing mIL-3Rα SP2 or SP1 and wild type (*wt*) β_{IL-3} or C73Aβ_{IL-3}. Cells were grown in a medium supplemented with various concentrations of mIL-3 as indicated for 2 days, and growth was measured in triplicate by [^{3}H]thymidine incorporation in at least two independent experiments. *B*, cell surface expression of wild type and C73Aβ_{IL-3} subunits as detected by flow cytometry following the method described under "Experimental Procedures." Cells stained with only the PE-conjugated antibody were used as a control for each assay (depicted by the *gray shaded area*).

and Fig. 5). High affinity binding with mIL-3Rα SP1 plus C73Aβ_{IL-3} was still present, although it was different from that of mIL-3Rα SP1 in the presence of wild type β_{IL-3}. Relative to the number of low affinity sites, there were fewer high affinity sites with mIL-3Rα SP1 plus C73Aβ_{IL-3} in either COS7 or CTLL2 cells, compared with mIL-3Rα SP1 plus wild type β_{IL-3}. Also, these sites were of higher apparent affinity, with K_d values of ~20–30 pM in CTLL2 and COS7 cells (Table 2 and Fig. 5*A*). This is quite analogous to our findings for hIL-3 binding to hβc C67A and hIL-3Rα SP1 (see above). Also analogous to our findings for hIL-3 binding to hβc C67A and hIL-3Rα SP2 (the α subunit variant lacking the N-terminal Ig-like domain), we observed complete loss of high affinity binding in mIL-3 binding experiments using mIL-3Rα SP2 and C73Aβ_{IL-3} (Table 2). High affinity binding in COS7 cells expressing mIL-3Rα SP2 and C73Aβ_{IL-3} was not detectable, in contrast to cells co-expressing mIL-3Rα SP2 and wild type β_{IL-3}, which gave a K_d of 738 pM (Table 2 and Fig. 5*A*), consistent with our previous findings (31).

The β_{IL-3} receptor offers an exceptional opportunity to examine direct engagement of mIL-3 by β_{IL-3}, thus enabling ligand binding to be examined in the absence of the mIL-3Rα subunit and without the need for any consideration of inter-subunit heterodisulfide formation. Consequently, direct binding of mIL-3 by the β_{IL-3} receptor can serve as a direct measure of the integrity of the ligand-binding epitope and allow assessment of whether mutation of the domain 1 D-E loop disrupts the ligand-binding site formed between domains 1 and 4 (25). Using cold saturation assays, a K_d value for direct binding between mIL-3 and the wild type β_{IL-3} receptor of 13.8 nM was determined (Table 2 and Fig. 5*A*), consistent with previous results (25, 40). In contrast, no direct mIL-3 binding was detectable in assays per-

TABLE 2
Dissociation constants for mIL-3 binding to cells expressing mIL-3Rα isoforms and wild-type or mutant C73AβIL-3 subunits

Cell line	mIL-3Rα	β_{IL-3}	High affinity K_d ± S.E.[a]	High affinity sites/cell ± S.E.	Low affinity K_d ± S.E.	Low affinity sites/cell ± S.E.	No. of experiments
			pM		*nM*		
COS7		Wild type			13.8[b]	2,767,952 ± 97,485	3
COS7		C73A			ND[c]		3
COS7	mSP1	Wild type	173 ± 292	8087 ± 1187	13.8[d]	1,076,673 ± 89,364	3
COS7	mSP1	C73A	33 ± 8	6831 ± 733	45[d]	291,007 ± 25,052	3
COS7	mSP2	Wild type	738 ± 125	115,863 ± 21,423	13.8[d]	1,204,400 ± 80,213	3
COS7	mSP2	C73A	ND				3
CTLL2	mSP1	Wild type	159 ± 138	186,609 ± 9001	—[e]		2
CTLL2	mSP1	C73A	10 ± 4.3	9613 ± 1333	45[d]	732,901 ± 84,130	2

[a] With the exception of direct binding assays, all binding was determined using the hot saturation binding assay, and K_d ± S.E. was determined by co-analysis of data from multiple experiments using LIGAND (39).
[b] For measurement of direct binding to β_{IL-3}, the cold saturation binding assay was used, and K_d ± S.E. was determined by co-analysis of data from multiple experiments using LIGAND (39).
[c] ND, no binding was detected above background.
[d] To improve the accuracy of determination of the high affinity K_d, the low affinity site K_d (attributable to direct binding by β_{IL-3}) was fixed at 13.8 nM. In the case of β_{IL-3} C73A, because direct binding was absent, the low affinity site K_d was fixed to that of mIL-3Rα SP1 (45 nM).
[e] A one-site model was statistically more significant than a two-site model.

formed using COS7 cells overexpressing the C73Aβ_{IL-3} mutant (Table 2). Considering our prior work establishing that the same residues within the elbow region formed between domains 1 and 4 of β_{IL-3} serve as the ligand-binding epitope for both direct mIL-3 binding by β_{IL-3} and for high affinity binding in the presence of mIL-3Rα SP2 (31), our results suggest that the β_{IL-3} domain 1 D-E loop disulfide is critical for the structural integrity of β_{IL-3} and that mutation of this loop disrupts the precise positions of key ligand-binding residues. The level of C73Aβ_{IL-3} cell surface expression was comparable with that of the wild type receptor in COS7 cells (Fig. 5*B*) as well as in CTLL2 cells (Fig. 4*B*). The results described above for the mIL-3Rα SP1 are consistent with our previous finding that high affinity binding with mIL-3Rα SP1 does not involve the critical IL-3 binding residues of the ligand-binding elbow region (25), so the binding is not abolished as it is with direct binding and high affinity binding with mIL-3Rα SP2.

Properties of a Mutant hIL-5Rα Receptor with No Free Cys in the Ectodomain—A free Cys in the ectodomains of each of the respective α receptors was postulated to interact with the hβc domain 1 D-E loop disulfide to form an interchain disulfide in the heterodisulfide model of receptor activation (32, 33, 41). The hIL-5Rα provides a favorable case for testing this hypothesis because Cys^{66} is the only free Cys residue in the extracellular domain of this receptor (42) and is located in D1, the proposed site of interaction with the hβc domain 1 D-E loop (33). hIL-5Rα has two conserved disulfides in the CRM. It also possesses a third pair of cysteines in domain 3 (D3), which behave as if they are a structural disulfide (42) and which align with the cysteines forming the disulfide between the B-C and F-G loops in domain 3 of IL-13Rα. Cys^{66} in hIL-5Rα was mutated to serine and stably transfected into CTLL2 cells expressing hβc. If Cys^{66} in hIL-5Rα were involved in the formation of a heterodisulfide with hβc, it would be expected that C66S hIL-5Rα would be unable to support growth signaling with a severe loss of function similar to that seen with C67Ahβc. In cell lines derived from two independent transfections, it was found that growth signaling was only slightly reduced with C66S hIL-5Rα compared with wild type hIL-5Rα in CTLL2 cells co-expressing wild type hβc (Fig. 6). Cys^{66} is known to be close to the IL-5 binding site in domain 1 because, when it is derivatized with isothiazolone derivatives, there is severe inhibition of IL-5 binding (42). However, C66S hIL-5Rα is still capable of forming a signaling complex with IL-5 when co-expressed with hβc. Our results clearly do not support the model that a free Cys residue in the hIL-5Rα ectodomain is required for receptor activation.

Cross-species Conservation of the Domain 1 D-E Loop Disulfide in the βc Receptor Family—We carried out an alignment of βc from different species using mRNA and genomic sequences from GenBank™ in order to examine the conservation of the domain 1 D-E loop disulfide. The aligned protein sequences show amino acid identities with hβc ranging from 57 to 97%. The structural disulfides linking β strands 1A and 1B and strands 1D and 1E plus those linking strands 3A and 3B and strands 3D and 3E are absolutely conserved as are the binding residues Tyr^{347}, Ile^{350}, and Tyr^{403} (numbered according to hβc). Also absolutely conserved are the WS*X*WS motif and the R*X*R*X*(R/K) of the Trp-Arg zipper in domain 4. The portion of the alignment covering the D-E loop disulfide is shown in Fig. 1*D*. Notably, the domain 1 D-E loop disulfide is present in all species examined, except horse, but the exact composition of the disulfide motif is variable and takes the forms C*XXXX*C, C*XXXXX*C, and C*XX*C. The absence of the disulfide motif from the horse βc domain 1 D-E loop provides further support for the notion that heterodisulfide formation with the α subunits is not a requisite step for receptor activation.

DISCUSSION

It is well established that the activation of hβc by IL-3, IL-5, or GM-CSF involves the formation of a heterodimer involving hβc and the respective cytokine-specific α receptors. An additional novel step in receptor activation involving the formation of a critical interchain disulfide between α and β receptors was proposed by Stomski *et al.* (32, 33) because it was observed that a small proportion of the α·hβc heterodimers were disulfide-cross-linked (32). A critical link between this disulfide cross-linking and receptor activation was suggested by the properties of hβc mutants in which the domain 1 D-E loop disulfide had been mutated. Stomski *et al.*

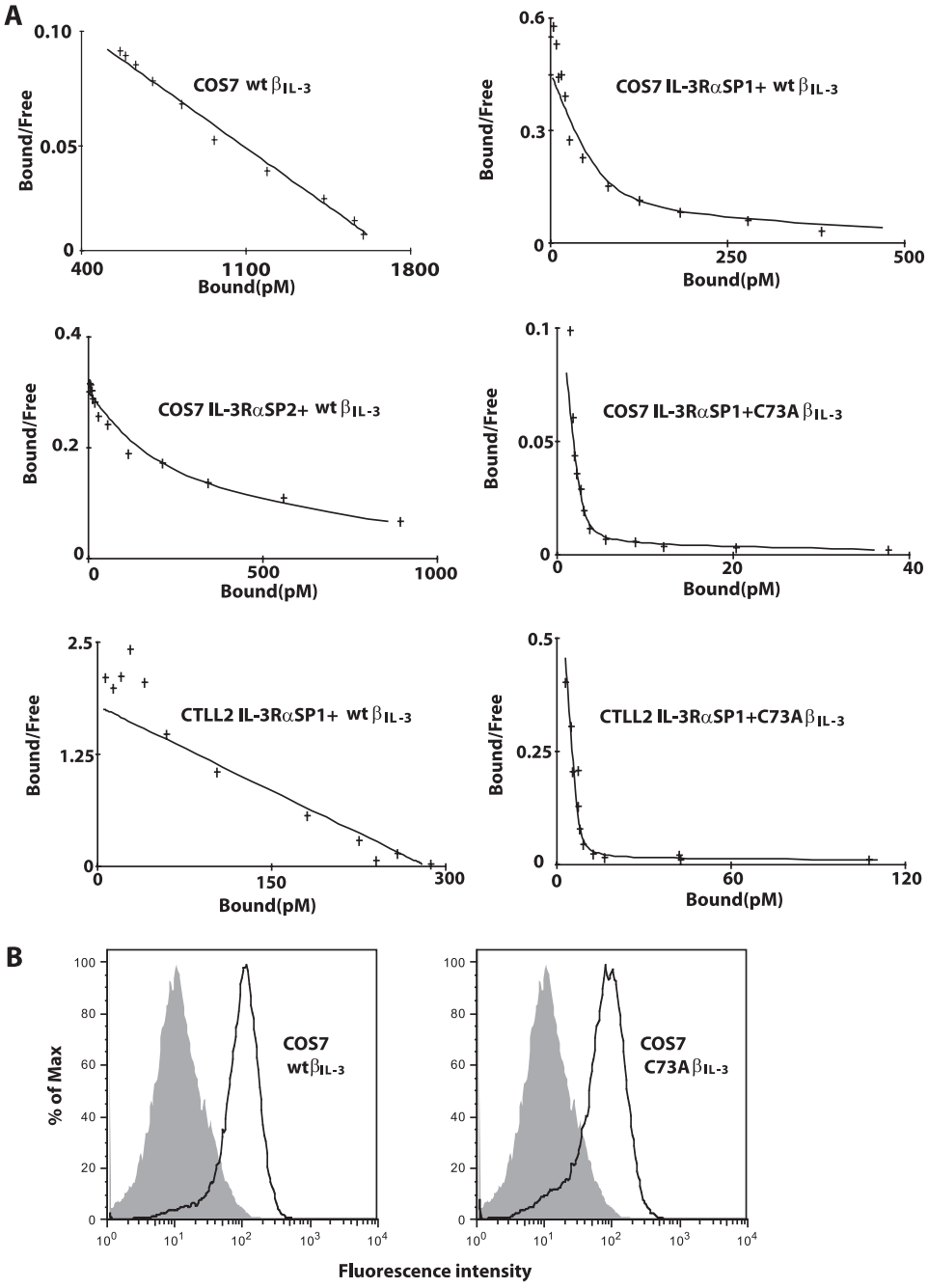

FIGURE 5. **mIL-3 binding properties of C73Aβ_{IL-3} and cell surface expression in COS cells.** *A*, Scatchard plots of ^{125}I-labeled mIL-3 binding data for COS7 and CTLL2 cells expressing wild type (*wt*) β_{IL-3} or C73Aβ_{IL-3} with or without mIL-3Rα mSP1 or mSP2. Each plot in the figure is labeled with the cells used followed by the receptors measured. The *top left panel* shows a representative cold saturation binding assay performed on COS7 cells expressing the wild type β_{IL-3} subunit alone. The remaining *panels* show plots of hot saturation binding assays for the different receptor combinations expressed in either COS or CTLL2 cells as indicated in the figure. In each plot, data from a representative hot or cold saturation binding experiment are shown with the line of best fit determined by co-analysis of data from several binding experiments using LIGAND (39); the derived K_d values are shown in Table 2. Curvilinear plots are indicative of two-site binding models, whereas one-site binding models show a linear fit to the data as shown in the *top left panel*. *B*, cell surface expression of wild type and mutant C73Aβ_{IL-3} subunits as detected by flow cytometry following the method described under "Experimental Procedures." Cells stained with only the PE-conjugated antibody were used as a control for each assay (depicted by the *gray shaded area*).

(33) reported that mutagenesis of either Cys residue of the domain 1 D-E loop disulfide of hβc abolished receptor activation without abolishing high affinity binding. The domain 1 D-E loop disulfide is well conserved in the hβc, mβc, and β_{IL-3} receptors (Fig. 1*D*) and is additional to the two structural disulfides present in the CRMs of these receptors. The results with the mutant receptors (33) suggested that the disulfide might have a unique non-structural role that involves a disulfide exchange with a free SH group in domain 1 of the α receptor, with the participating Cys residues being brought into close proximity in the high affinity complex. Implicitly, this hypothesis presumes that there will be no structural perturbation caused by mutation of the β subunit domain 1 D-E loop disulfide that could explain the loss of receptor activation.

The present work aimed to clarify the role of the hβc domain 1 D-E loop disulfide in receptor activation. Recent structural and biochemical studies (23–26) have revealed that residues within the hβc domain 1 contribute to the cytokine binding epitope and suggest an alternative model by which mutation of the domain 1 D-E loop disulfide might disrupt the three-dimensional positions of key ligand-binding residues in domain 1, thus compromising ligand binding and receptor activation. We thoroughly investigated the role of the domain 1 D-E loop disulfide in hβc and β_{IL-3} high affinity binding and receptor activation. We included the β_{IL-3} receptor, which is closely related to hβc, as well as IL-3Rα SP2, a new isoform of IL-3Rα, in the study.

Disruption of the domain 1 D-E loop disulfide gave complete abolition of growth signaling in CTLL2 cells expressing either the hβc or β_{IL-3} receptor in combination with any of the available α receptors, including IL-3Rα SP2, a splice variant that lacks domain 1 of the α receptor. Using the C67Ahβc mutant, in which the key disulfide was disrupted, we verified the previous finding (33) of retention of high affinity binding with hIL-3Rα SP1 and hGM-CSFRα. However, the hIL-3 and hGM-CSF binding characteristics of the C67A mutant hβc when co-expressed with hIL-3Rα SP1 and hGM-CSFRα, respectively, were noticeably different from those of the wild type receptor counterparts. There were far fewer high affinity sites for

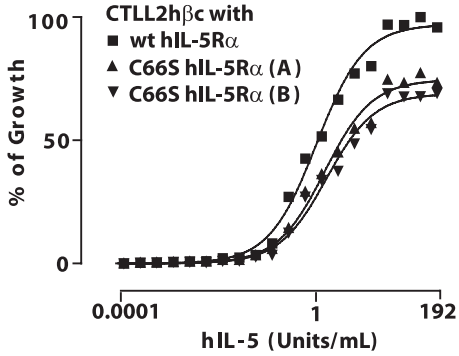

FIGURE 6. **Proliferation of CTLL2 cell lines stably co-expressing hβc and wild type or C66S hIL-5Rα.** Plots depict the percentage of growth in CTLL2 cells stably expressing wild type (*wt*) or mutant receptors in response to a serial dilution of hIL-5. The data shown are representative of data from at least two experiments performed on two independently transfected stable CTLL2 cell lines (both are shown, denoted *A* and *B*).

both hIL-3 and hGM-CSF when hβc domain 1 D-E loop disulfide mutants were co-expressed with the hIL-3Rα SP1 and hGM-CSFRα, respectively, compared with the wild type counterparts, despite comparable levels of cell surface expression. This trend was also seen in the data obtained by Stomski *et al.* (33), although it was attributed to receptor aggregation. The binding also appeared to be of higher affinity in the previous study (33).

We believe that the abnormal high affinity binding properties of the C67Ahβc subunit described above are due to a loss of structural integrity of the receptor. Unequivocal verification of structural perturbation of this receptor resulting from loss of the domain 1 D-E loop disulfide was obtained from our studies with the hIL-3Rα SP2 splice variant. hIL-3Rα SP2 could bind hIL-3 with high affinity in the presence of the wild type hβc subunit, but no high affinity binding was detectable with the C67A mutant hβc. These data provide clear support for the hypothesis that mutating the hβc domain 1 D-E loop disulfide bond causes structural distortion of the hβc ligand-binding epitope. It is also notable that the hIL-3Rα SP2 isoform is a naturally occurring splice variant that lacks D1, the domain attributed the function of mediating the heterodisulfide cross-link with the hβc domain 1 D-E loop cysteines in the original receptor activation model (33). Thus, the fact that wild type hβc and wild type hIL-3Rα SP2 subunit can bind hIL-3 with high affinity and initiate signaling argues against the necessity of intersubunit cross-linking as a step in IL-3 receptor activation.

Further compelling evidence that mutation of the domain 1 D-E loop causes a structural perturbation came from extension of our study to the related mouse IL-3-specific receptor, β_{IL-3}. Studies with C73Aβ_{IL-3} (the mutation analogous to C67A in hβc) in the presence of the mIL-3Rα SP1 or SP2 subunits gave findings analogous to those in our studies of their human receptor counterparts. For instance, in the case of mIL-3Rα SP1 and C73Aβ_{IL-3}, high affinity binding was retained, although it was abnormal, with a low number of high affinity ligand-binding sites. In contrast, no binding was detected when C73Aβ_{IL-3} was coexpressed with the mIL-3Rα SP2 isoform. The β_{IL-3} receptor provides a unique opportunity for the direct interaction with mIL-3 to be examined in the absence of any mIL-3Rα subunit because β_{IL-3} binds mIL-3 directly with a K_d of ~20 nM. Strikingly, in complete contrast to wild type β_{IL-3}, the C73Aβ_{IL-3} mutant receptor did not detectably bind mIL-3 in cold saturation assays, providing unequivocal evidence that disruption of the β_{IL-3} domain 1 D-E loop compromises structural integrity within domain 1 of the receptor. It is probable that this mutation causes a distortion of the β_{IL-3} domain 1-domain 4 ligand-binding epitope (25), thereby accounting for the resultant loss of direct mIL-3 binding. This epitope is also critical for high affinity binding with mIL-3Rα SP2 (31), explaining the complete loss of high affinity binding with this isoform. In contrast, high affinity binding with mIL-3Rα SP1 is not dependent on this epitope, but the low number of high affinity sites observed when this mIL-3Rα isoform and C73Aβ_{IL-3} are co-expressed provides evidence that this mutation causes a loss of β_{IL-3} structural integrity.

The above findings are most consistent with a revised model for receptor activation where, rather than an α subunit forming a heterodisulfide with the β subunit domain 1 D-E loop disulfide as a step in receptor activation, the β subunit domain 1 D-E loop plays a crucial role in maintaining the position(s) of a key ligand-binding residue(s) in domain 1, with mutation of the D-E loop cysteines compromising ligand recognition and receptor activation. In addition, our finding that a mutant hIL-5Rα possessing no free Cys in its ectodomain still signals effectively is also contrary to the α·β heterodisulfide receptor activation model. The confusion over the role of the hβc domain 1 D-E loop disulfide has arisen largely because of the retention of high affinity binding with some α receptors when Cys^{62} or Cys^{67} is mutated to disrupt this disulfide bond. Normally, mutation of a structural disulfide would result in complete loss of binding. However, in the case of the hβc domain 1 D-E loop disulfide mutants, high affinity binding is abolished in some receptor combinations but remains detectable in others, albeit with a concomitant large reduction in the number of binding sites relative to the wild type receptor. The hβc or β_{IL-3} domain 1 D-E loop disulfide is well positioned to influence the key functional epitope residues in the ligand binding elbow region formed between domains 1 and 4 of hβc or β_{IL-3}, particularly those in the domain 1 A-B and E-F loops (Fig. 1*C*).

The differential effects observed for ligand binding by hβc and β_{IL-3} when co-expressed with the full-length IL-3α (SP1) or the splice variant lacking the N-terminal Ig-like domain (SP2) are consistent with our prior work suggesting that the two IL-3α isoforms dictate which of two distinct IL-3 binding interfaces on the β receptors are used (25, 31, 47). It is clear that the ligand-binding epitopes on hβc and β_{IL-3} utilized by the IL-3·IL-3Rα SP2 complex are the most affected by disruption of the domain 1 D-E loop disulfide. After considering the recent findings that domains 1 of hβc and β_{IL-3} form an essential part of the functional epitopes for ligand binding in the respective activation complexes and that the N-terminal Ig-like domain of the IL-3Rα subunits is dispensable for receptor activation, we present a revised model to reconcile why mutation of the hβc and β_{IL-3} domain 1 D-E loop disulfides abrogates receptor activation. Our data pro-

vide clear support for the hypothesis that this important disulfide is critically important to maintaining the correct three-dimensional positions of the ligand-binding residues at the domain 1-domain 4 interface in hβc and β_{IL-3}, which is imperative for optimal ligand recognition and assembly of the receptor subunits within the high affinity complex in the correct orientation to facilitate receptor signaling.

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