Suppression of Inhibin A Biological Activity by Alterations in the Binding Site for Betaglycan*

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Inhibins A and B negatively regulate the production and secretion of follicle-stimulating hormone from the anterior pituitary, control ovarian follicle development and steroidogenesis, and act as tumor suppressors in the gonads. Inhibins regulate these reproductive events by forming high affinity complexes with betaglycan and activin or bone morphogenetic protein type II receptors. In this study, the binding site of inhibin A for betaglycan was characterized using inhibin A mutant proteins. An epitope for high affinity betaglycan binding in inhibin A for betaglycan was characterized using inhibin A protein type II receptors. In this study, the binding site of inhibin A for betaglycan was characterized using inhibin A mutant proteins. An epitope for high affinity betaglycan binding in inhibin A for betaglycan was characterized using inhibin A protein type II receptors. In this study, the binding site of inhibin A for betaglycan was characterized using inhibin A mutant proteins. An epitope for high affinity betaglycan binding was detected spanning the outer convex surface of the inhibin α-subunit. Homology modeling indicates that key α-subunit residues (Tyr50, Val108, Thr111, Ser112, Phe118, Lys119, and Tyr120) form a contiguous epitope in this region of the molecule. Disruption of betaglycan binding by the simultaneous substitution of Thr111, Ser112, and Tyr120 to alanine yielded an inhibin A variant that was unable to suppress activin-induced follicle-stimulating hormone release by rat pituitary cells in culture. Together these results indicate that a high affinity interaction between betaglycan and residues Val108–Tyr120 of the inhibin α-subunit mediate inhibin A biological activity.

Inhibin A and inhibin B, members of the TGFβ2 superfamily, are essential regulatory factors in mammalian reproduction. Their expression is critical for the regulation of fertility based on their dual inhibitory actions on follicle-stimulating hormone (FSH) secretion by the pituitary (1, 2) and gametogenesis in the gonads (3, 4). It is recognized that inhibins regulate these reproductive events by inhibiting the stimulatory actions of the structurally related proteins, activins (5, 6). Inhibins are heterodimers of an 18-kDa α-subunit disulfide-linked to one of two 13-kDa β-subunits (βA and βB), resulting in inhibin A or inhibin B, respectively. Each activin is composed of two β-subunits; βA-βA (activin A), βA-βB (activin AB), and βB-βB (activin B). Although the inhibin A and inhibin B crystal structures have not been determined, based upon sequence identity it is expected that the β-subunits would retain a similar conformation to that observed in the activin A dimer (7). Thus, within either β-subunit, two pairs of anti-parallel β-strands, forming a short and a long “finger,” stretch outward from the cysteine knot core of the monomer. At the base of the fingers, each β-subunit forms an α-helix, which together with the prehelix loop, generates the “wrist” region of the molecule (8).

An interchain disulfide bond between Cys80 of the βA-subunit (Cys79 of the βB-subunit) and Cys95 of the α-subunit covalently connects the two chains of the inhibin dimers. In humans, unlike other species, two molecular mass isoforms of mature inhibin A and B (31 and 34 kDa) have been identified (9). This molecular mass heterogeneity is due to the presence of two N-linked glycosylation sites at Asn36 and Asn70 of the α-subunit. Asn36 is always glycosylated (producing 31-kDa inhibin A or B), whereas glycosylation of Asn70 is differentially regulated (producing 34-kDa inhibin A or B) (10). The presence of a second carbohydrate moiety significantly reduces the biological activity of inhibin A (9), suggesting that human inhibins may be under more stringent control than inhibins from other species.

Inhibins exert their biological effects by antagonizing the actions of TGFβ ligands that utilize activin (ActRII/IIB) or BMP (BMPRII) type II receptors as part of their signaling complex (11). The inhibin residues involved in binding to the type II receptors can be inferred from the crystal structure of activin A bound to ActRIIB (12). In this structure, the receptor makes contact with the outer convex surface of the finger regions of activin. The activin:ActRIIB interface involves hydrophobic (Ile30, Ala31, Pro32, Pro86, Leu92, Tyr94, and Ile100) and ionic/polar (Arg87, Ser90, Lys103, and Glu111) residues on the βA-subunit. The affinities of the inhibin isoforms for ActRII/IIB are too low to facilitate functional antagonism of activin signaling, suggesting the requirement for a co-receptor to potentiate inhibin actions. Indeed, recent studies have shown that the biological effects of inhibin A are dependent upon interactions with betaglycan, a cell surface proteoglycan that also acts as a TGFβ2 co-receptor (13). Betaglycan binds inhibin A directly and promotes the formation of a stable high affinity complex involving activin or BMP type II receptors. Sequestration of type II receptors in this way prevents their interactions with signaling ligands such as activins or BMPs.

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§ The abbreviations used are: TGF, transforming growth factor; FSH, follicle-stimulating hormone; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high pressure liquid chromatography; BMP, bone morphogenetic protein.
known about the regions of inhibin involved in this interaction. Activins do not bind betaglycan, suggesting that binding is mediated by the α-subunit of inhibin. The human inhibin α-subunit is synthesized as a precursor consisting of a 43-amino acid Pro region, a 171-amino acid αN region, and a 134-amino acid αC region (15) (see Fig. 2A). In this study, we have identified residues within the mature (αC) region of the inhibin α-subunit essential for binding betaglycan. Mutation of these residues generated inhibin A variants with dramatically reduced biological activity. In addition, we have shown that the binding epitope on inhibin A for betaglycan is conserved across the TGFB isoforms, providing important information regarding TGFB receptor assembly.

**EXPERIMENTAL PROCEDURES**

Production of Inhibin A Mutants—An overlapping PCR strategy was utilized to incorporate mutations in the mature region of the full-length human inhibin α-subunit cDNA. Gel-purified PCR products were digested with HindIII and EcoRI and then subcloned into HindIII/EcoRI-digested pcDNA3.1 (Invitrogen). For each construct, the mutated N-terminal mature region was confirmed by DNA sequencing.

Production of mutant inhibins was achieved using Lipofectamine (Invitrogen) to transiently transfet CHO cells. Briefly, CHO cells were plated at 1 × 10⁶ cells/well in a 6-well plate. Wild type or mutant α-subunit DNA (1.6 μg) was combined with 3.3 μg of βA-subunit DNA, and Lipofectamine was added according to the manufacturer’s instructions. After 20 min of incubation, DNA-Lipofectamine complexes were added directly to the plates, which were incubated in serum-free Opti-MEM medium for a further 48 h at 37 °C in 5% CO₂.

Inhibin A ELISA—The inhibin A ELISA (Diagnostic Systems Laboratories, Webster, TX) was used as described (16) employing kit reagents provided by Oxford Bio-innovation Ltd. (Upper Heyford, UK). The ELISA utilized the βA-subunit antibody (E4) as capture antibody and α-subunit antibody (R1) as label. Inhibin A immunoaggregate reaction (91/624) was used as a reference preparation. The sensitivity of the assay was 2 pg/ml. The between assay variation was 13.1% (n = 4). The mean index of precision (α) was 0.048.

**Stable Cell Lines**—Inhibin A bicistronic expression vectors encoding the human cDNA for the α- and βA-subunits were constructed using the pIRES plasmid (Clontech, CA). The pIRES plasmid ensures enhanced transcription and translation of the inhibin α-subunit cDNA cloned into the first multiple cloning site over the production of the βA-subunit in the second cloning site, allowing for the preferential production of inhibin A rather than activin A (17). Wild type or selected mutant inhibin α-subunit cDNAs, amplified from pcDNA3.1-inhibin α vectors, were inserted into the first multiple cloning site at the NheI/EcoRI sites of pIRES. The cDNA for the full-length inhibin βA-subunit was amplified from pcDNA3.1-βA vector and inserted into the XbaI/NotI sites of the second multiple cloning site of pIRES.

CHO cells were transfected with the pIRES-Inhibin A vectors, and cells stably expressing wild type or mutant inhibin A were selected with 500 μg/ml G418. Five or more clones for the expression of each construct were isolated. To produce wild type and mutant inhibin A, stable CHO cell lines were grown to confluence, and then the medium was changed to serum-free Opti-MEM. After 72 h, the conditioned medium was collected and concentrated using Amicon Ultra centrifugal filter units (Millipore, Billerica, MA). Concentrated protein was applied to a HiLoad 16/60 Superdex 200 size exclusion column (GE Healthcare), and the proteins were eluted in 0.1 M phosphate, pH 7.4. The inhibin A ELISA, in combination with Western blot analysis using a monoclonal antibody against human inhibin α-subunit (R1), identified fractions containing 31-kDa inhibin A. These fractions were pooled and purified by immunoaffinity chromatography with R1 antibody immobilized to an Affi-Gel-Hz support (Bio-Rad). Bound protein was eluted with guanidinium HCl, and 31-kDa inhibin A was further purified on a reverse phase HPLC-solid phase extraction (Vydac) column in 0.1% trifluoroacetic acid, 60% acetonitrile.

**Betaglycan Binding Assay**—To screen the large number of inhibin A mutants, a high throughput betaglycan binding assay was developed (9). Briefly, COS7 cells were plated at 1 × 10⁵ cells/well in 24-well plates. The following day, the cells were transfected with 15 ng of vector (pcDNA3.1) control or betaglycan cDNA using the Lipofectamine transfection reagent and incubated for 48 h at 37 °C. To ensure uniform expression of betaglycan, a cell surface ELISA was performed, as previously described (14). The cells were washed in binding buffer (DMEM + 0.1% bovine serum albumin). Binding buffer (200 μl) or increasing concentrations of wild type or mutant inhibin A conditioned medium (1–200 pM) were added to the wells, together with [125I]inhibin B (25 μl, 40,000 cpm/well). The differential affinity of the inhibin forms for betaglycan (9) ensured that very low amounts of inhibin A (IC₅₀ 1.2 pm) were required to displace [125I]-inhibin B. The plates were incubated for 3 h at room temperature, and the cells were washed in phosphate-buffered saline and solubilized in 1% Triton X-100. Radioactivity was measured using a gamma counter. The binding data were analyzed using the Prism program (version 2.0 from GraphPad Software, San Diego, CA).

**ActRII Binding Assay**—To ensure that point mutations that disrupted inhibin A binding to betaglycan did not affect overall protein structure, we utilized an activin type II receptor binding assay. Selected inhibin A mutants were assessed for their ability to displace [125I]-inhibin A from 293T cells expressing ActRIIB, as previously described (18).

**Inhibin A in Vitro Bioassays**—Wild type and mutant inhibins were assessed for their ability to suppress the release of FSH by rat pituitary cells in culture or a mouse pituitary gonadotrope cell line (LBT2). The rat pituitary cultures were performed as previously described (19) except FSH release rather than FSH content was used as assay end point. LBT2 cells were plated in 48-well plates at a density of 250,000 cells/well. The cells were allowed to recover for 24 h in DMEM supplemented with 10% fetal calf serum. The cells were then washed with DMEM + 0.2% fetal calf serum and treated with increasing doses of inhibin variants for 24 h in the same medium. FSH levels were determined by a specific rat FSH immunofluorometric assay as previously described (20) employing reagents kindly provided by A. Groothuis and J. Verhagen of (N.V. Organon). The sensitivity of the assay was 12.5 pg/well (human inhibin A
affinity for betaglycan 20-fold (9); and (ii) finger 2 (Gly101–(Fig. 1), based on the fact that glycosylation of Asn70 reduces
sequent mutations were made in the context of this construct. We initially mutated the inhibin
complexity of the samples to be analyzed, and all subse-
ensure that only 31-kDa inhibin A was produced. This reduced
potential betaglycan-binding sites: (i) the wrist (Gly64–Pro85)
 instances where multiple residues were mutated in the one variant. Residues that when mutated resulted in a
4-fold reduction in inhibin A affinity for betaglycan are shown in
bold. The inhibin α-subunit sequence is aligned with the TGFβ isoforms, which are also known to utilize betaglycan as a co-receptor. The numbering is according to the mature human inhibin α-subunit sequence.

immunoreactive preparation). The between assay variation
based on the repeated measurement of a purified inhibin pre-
paration was 18.7% (n = 8). The mean index of precision (λ) was
-0.078.

Homology Modeling of Inhibin A—The inhibin dimer was
homology modeled using coordinates from the Protein Data
Bank. The inhibin α-subunit was modeled from Protein Data
Bank code 2QCQ (21), whereas for the inhibin βA-subunit the
coordinates from Protein Data Bank code 2ARV (7) were uti-
liized. In each case, Swiss model was used to perform the mod-
eling. The dimer was made by superposing the coordinates of
the inhibin α- and βA-subunits onto the dimer of Protein Data
Bank code 1KTZ using secondary structure matching (22) as
implemented in COOT (23). The figures were produced using
PYMOL (24).

RESULTS

Selection of Inhibin A Residues for Mutation—In humans,
two molecular mass isoforms of mature inhibin A (31 and 34
kDa) have been identified (10). This molecular mass heteroge-
neity is due to differential glycosylation of Asn70 of the α-sub-
unit. We initially mutated the inhibin α-subunit (N70Q) to
ensure that only 31-kDa inhibin A was produced. This reduced
the complexity of the samples to be analyzed, and all subse-
quent mutations were made in the context of this construct.

Two regions of the inhibin α-subunit were identified as
potential betaglycan-binding sites: (i) the wrist (Gly64–Pro85)
(Fig. 1), based on the fact that glycosylation of Asn70 reduces
affinity for betaglycan 20-fold (9); and (ii) finger 2 (Gly101–
Leu127) (Fig. 1), because polyclonal antibody 1989 (directed
against amino acids 109–122) immunoneutralized inhibin A
bioactivity (25). Residues through these regions, together with
selected amino acids in finger 1 (Phe39–Ser53) of the inhibin
α-subunit, were substituted with alanine using in vitro
mutagenesis. In all, a set of 41 variants mutated at 50 different
positions were generated (Fig. 1).

Synthesis and Secretion of Mutant Polypeptides—The mutant
proteins were expressed in CHO cells and a set of 36 variants
forms produced by CHO cells (Table 1). In contrast, mutation
of residues through the fingers of the α-subunit typically
reduced inhibin levels by 50–75%, although the ratio of mature
to high molecular forms of dimeric inhibin was maintained
(Table 1 and Fig. 2B, lanes 6, 8, and 9). Interestingly, five point
mutations in the fingers of the α-subunit (F39A, I48A, P51A,
L106A, and V108A) completely abrogated inhibin A expression
(Fig. 2B, lanes 3, 5, and 7, and data not shown), suggesting that
these hydrophobic residues are required for correct folding and
dimerization with the β-subunit.

Betaglycan Binding of Inhibin A Mutants—Based on the
structural location of the mutated α-subunit residues, the
inhibin A variants generated in this study were separated into
three groups and assessed for their ability to displace 125I-in-
bolin B from betaglycan in a radioligand binding assay. The
differential affinity of the inhibin forms for betaglycan (9)
ensured that very low amounts of inhibin A (IC50 = 1.2 pm)
were required to displace 125I-inhibin B from transfected COS7
cells. Nontransfected COS7 cells, which do not bind inhibin A
or B (9), were used as controls in the binding assays. Group A
residues were those at the N terminus and in finger 1, and, in
general, mutation of these residues had little effect on betagly-
can binding (Fig. 3A and Table 1). An exception was the Y50A
α-subunit mutant, which displayed a 4-fold reduction in affinity
for betaglycan (IC50, 48 pm) compared with wild type inhibin A
(IC50, 1.2 pm). It is worth noting that mutants F39A, I48A, and
P51A were not expressed, and so the contribution of these resi-
dues to betaglycan binding could not be determined.

Group B residues were those in close proximity to the second
glycosylation site in the inhibin α-subunit (Asn70). Because the
attachment of a carbohydrate moiety at this site significantly
reduces the affinity of inhibin A for betaglycan (9), it was antici-
pated that juxtaposed residues would have an important role in
betaglycan binding. However, despite all of these mutants
(G64A/L65A, H66A/L67A, P68A/P69A, L71A/S72A, L73A/
V75A, A78G/P82A, and Y86A/P90A) having multiple residues
substituted to alanine, there was only a limited effect on the
affinity of inhibin A for betaglycan (Fig. 3B and Table 1). This

FIGURE 1. Sequence alignment of the inhibin α-subunit and TGFβ isoforms. Inhibin α-subunit residues deleted (Δ4–25) or substituted in this study are shaded black. A line above the substituted residues indicates
instances where multiple residues were mutated in the one variant. Residues that when mutated resulted in a
>4-fold reduction in inhibin A affinity for betaglycan are shown in
bold. The inhibin α-subunit sequence is aligned with the TGFβ isoforms, which are also known to utilize betaglycan as a co-receptor. The numbering is according to the mature human inhibin α-subunit sequence.
suggests that the wrist region of the inhibin α-subunit is not part of the high affinity binding epitope for betaglycan and indicates that the presence of a carbohydrate group at Asn70 must affect betaglycan binding to a more distant portion of the α-subunit.

Group C mutants targeted residues in finger 2 of the α-subunit. Mutation of these residues had profound effects on betaglycan binding (Fig. 3C and Table 1). In particular, simultaneous substitution of Lys119 and Tyr120 reduced inhibin affinity for betaglycan 25-fold (IC50 7 pM). Other double mutations through this region, including R109A/T110A, T111A/S112A, and S117A/F118A, were also disruptive for betaglycan binding. Interestingly, Val108, which is essential for the dimerization of inhibin A (Fig. 2), also appears to be part of the epitope for high affinity betaglycan binding. Conservative substitutions at this site, as opposed to the introduction of alanine, were not disruptive for inhibin A dimer formation. Mutant V108L was expressed at levels comparable with other finger 2 variants but had a 6-fold lower affinity for betaglycan (IC50 7 pM) than wild type inhibin A. Together these results indicate that betaglycan primarily contacts inhibin A residues in the finger regions of the α-subunit.

Synergistic Effect of Combined Amino Acid Substitutions in Inhibin A Mutants—The inhibin A double mutant, K119A/Y120A, had a significantly reduced (25-fold) affinity for betaglycan compared with the wild type protein. A comparison of the single point mutations indicated that Tyr120 was the major determinant for betaglycan binding; mutation of Lys119 has only a marginal effect on inhibin A affinity (Fig. 3D and Table 1). The fact that simultaneous mutation of the two residues exacerbated the reduction in receptor binding suggested that together they formed part of the high affinity betaglycan-binding site on inhibin A.

To further reduce inhibin binding to betaglycan, the Y120A variant was combined with other disruptive finger 2 mutations. Given the importance of finger 2 residues in the correct folding and secretion of the inhibin A dimer (Fig. 2), it was not surprising that a majority of the combined mutants were expressed poorly or not at all (Table 1). However, several mutants were generated that had additive (V108L/K119A/Y120A) or synergistic (T111A/S112A/K119A/Y120A) effects. The T111A/S112A/K119A/Y120A variant was of particular interest because it was highly limited in its ability to displace 125I-inhibin B from binding to betaglycan (Fig. 3D and Table 1).

Purification and Receptor Binding of Inhibin A Mutants—Unfractionated conditioned medium was used in the initial assessment of betaglycan binding of the inhibin A mutants. Based on this binding assay, two inhibin A variants (K119A/
Y120A and T111A/S112A/Y120A) were selected for further analysis. Stable CHO cell lines expressing wild type or mutant inhibin A were generated. The secreted 31-kDa dimer was purified from 500 ml of conditioned medium using a combination of gel filtration, immunoaffinity chromatography, and reverse phase HPLC (Fig. 4). This procedure yielded 31-kDa inhibin A free of contaminating activin, as determined by Western blot and an activin A ELISA (data not shown). Quantitation of the inhibin A variants was determined using a specific inhibin A ELISA (Table 1). To confirm the data obtained with conditioned medium, the betaglycan binding activities of the purified inhibin A variants were studied. Wild type inhibin A readily displaced 125I-inhibin B (IC50 = 3.7 pm) from COS7 cells expressing betaglycan (Fig. 5A). In contrast, the K119A/Y120A (IC50 = 100 pm) and T111A/S112A/Y120A (IC50 = 720 pm) mutants had significantly reduced affinity for betaglycan. Subsequently, we assessed the ability of purified inhibin variants to bind to the activin type II receptor, ActRIIB (Fig. 5B). Wild type and mutant isoforms of inhibin A displayed similar affinities for ActRIIB, indicating that the mutations introduced into finger 2 of the α-subunit did not affect the overall structure of the inhibin A dimer.

**FIGURE 3.** Betaglycan binding of inhibin A mutants. COS7 cells were transfected with pcDNA3.1 (control) or betaglycan cDNA and 48 h later were subjected to competition binding as described under “Experimental Procedures.” Displacement curves for inhibin A variants with mutations at the N terminus/finger 1 (A), the wrist region (B), and finger 2 (C) of the α-subunit are shown. The effects of combined amino acid substitutions on the ability of inhibin A variants to bind betaglycan are also shown (D). The displacement curve generated in the presence of wild type inhibin A is shown for comparison (A–D). 125I-Inhibin B binding and displacement from nontransfected COS7 cells is also shown (A). The dashed lines represent the IC50 value for the displacement of 125I-inhibin B from COS7 cells. The amount of bound 125I-inhibin B was determined in triplicate for each dilution, and the values are the means ± S.D. Each inhibin A variant was assessed three to five times.

**FIGURE 4.** Purification of inhibin A variants. Conditioned medium from CHO cells stably expressing wild type inhibin A was concentrated and separated by size exclusion chromatography as described under “Experimental Procedures.” Inhibin A was detected by specific ELISA and by Western analysis using antisera to the α-subunit (R1). Fractions 25–27, containing mature inhibin A, were pooled and purified to homogeneity by a combination of immunoaffinity and reverse phase HPLC. The KY and TSY inhibin A variants were purified in the same manner. The yields of the inhibin A variants were −30–100 µg/liter conditioned medium.
Biological Activity of Inhibin A Mutants—In response to endogenous activin, \( \text{L}^\beta \text{T2} \) pituitary gonadotrope cells synthesize and secrete FSH (26, 27). Previous studies have shown that inhibin A can suppress FSH release from these cells (9, 28). The \( \text{L}^\beta \text{T2} \) gonadotrope cell system was used to assess the biological activity of the inhibin A variants. Wild type inhibin A suppressed FSH release by \( \text{L}^\beta \text{T2} \) cells in a dose-dependent manner (IC\(_{50}\) 85 pM) (Fig. 6A). In contrast, the \( K_{119}/Y_{120} \) variant, which displayed a 25-fold lower affinity for betaglycan than wild type inhibin A, had a reduced potency (6.5-fold) in this assay. Substantial disruption of betaglycan binding by the simultaneous substitution to alanine of \( \text{Thr}_{111}, \text{Ser}_{112}, \) and \( \text{Tyr}_{120} \) resulted in a further marked loss of in vitro activity (>70-fold). Similar results were obtained using the more physiologically relevant rat pituitary cell culture system, where wild type inhibin A (IC\(_{50}\) 25 pM) was 4-fold more potent than the \( K_{119}/Y_{120} \) variant (IC\(_{50}\) 100 pM) and at least 40-fold more potent than the \( T_{111}/S_{112}/Y_{120} \) variant (Fig. 6B). Together these results indicate that inhibin A biological activity is dependent upon a high affinity interaction with its co-receptor, betaglycan.

Location of Betaglycan Binding Epitope—The three-dimensional structure of inhibin A has yet to be determined. There-
fore, we constructed a homology model of the inhibin α-subunit (Fig. 7A) based on the activin A, BMP-3, and BMP-6 structures (7, 12, 21). Despite the relatively low sequence homology (25–35%) of these TGF/β family members, seven cysteine residues can be aligned and are thought to confer the same overall conformation in the α-subunit as observed in the activin and BMP structures. Thus, within the α-subunit, two pairs of anti-parallel β-strands, forming first a short and then a long finger, stretch outward like wings from the cysteine knot core of the monomer (Fig. 7A). The characteristic curvature of these fingers creates concave and convex surfaces through this region. At the opposite end of the molecule, a high proline region (8 of 24 residues) would ensure that the inhibin α-subunit has a truncated α-helix relative to other TGF/β family members.

Mapping of the inhibin α-subunit residues mutated in this study onto the modeled structure of inhibin A is shown in Fig. 7A. The determinants for betaglycan interaction (Tyr50, Val108, Arg109, Thr110, Thr111, Ser112, Ser117, Phe118, Lys119, and Tyr120) form a contiguous epitope on the outer convex surface of the fingers (Fig. 7A, cyan shading). Based on the amino acids present in this region, it appears that the binding of inhibin A to betaglycan is mediated primarily by ionic interactions. Val108, and to a lesser extent Phe118, were the only hydrophobic residues that when mutated, significantly decreased inhibin A affinity for betaglycan (Table 1). However, it is possible that some of the major binding determinants are not represented in the present series of inhibin A variants. The missing determinants are possibly among the residues, which when mutated were not expressed. Of these residues, Ile48 and Pro51 (Fig. 7A,

**FIGURE 7. Homology modeling of inhibin A.** A, ribbon schematic of a homology model of the inhibin heterodimer. The inhibin α-subunit is colored orange, whereas the inhibin βA-subunit is green. Key features of the inhibin α-subunit have been highlighted, and the side chain is indicated. Residues that play a role in binding to betaglycan are colored cyan. Other residues that are in close proximity to the betaglycan binding interface, but could not be mutated, are shown in gray. Asparagine residues of key glycosylation sites are colored red. B, ribbon schematic of the TGFβ3/TβRII complex. Coordinates from Protein Data Bank code 1KTZ (29). TGFβ3 is colored green with residues predicted to be involved in binding betaglycan indicated as side chains and highlighted in cyan. TβRII is colored pink.
Betaglycan-binding Site on Inhibin A

gray shading) are in close proximity to the betaglycan-binding site.

The three TGFβ isoforms, TGFβ1–3, also utilize betaglycan as a co-receptor. Sequence alignment indicates that several residues (Pro19 and Lys119) implicated in inhibin binding to betaglycan are identical in the TGFβ isoforms (Fig. 1). At other positions in the binding epitope (Val108 and Ser117), conservative amino acid substitutions are noted (Fig. 1). Importantly, these TGFβ residues (Fig. 7B, cyan shading) have not been implicated in type I and type II receptor interactions (29). In contrast, the corresponding residues in the activin βA- and βB-subunits are involved in binding to type II receptors (8) and, accordingly, bear limited homology to the inhibin α-subunit residues involved in binding betaglycan (data not shown).

DISCUSSION

To maintain gametogenesis, inhibin A and inhibin B are secreted by the gonads and act at the pituitary to regulate FSH production (2, 30). In this study, we show that the high affinity interaction of inhibin A with betaglycan, necessary for biological activity, is mediated by residues on the outer convex surface of the α-subunit. These results provide novel insights into the mechanism of action of inhibin A, which may be generally applicable to other members of the TGFβ superfamily.

Upon secretion, betaglycan binds inhibin A directly and promotes the formation of a stable high affinity complex involving activin B type II receptors (11, 13). Sequestration of type II receptors in this way prevents their interactions with signaling ligands such as activin A and activin B (13). To provide a structural basis for understanding the critical role betaglycan plays in facilitating the antagonistic actions of inhibin A, α-subunit mutants were generated and assessed for their ability to bind betaglycan. Mutagenesis of residues in the “fingers” of the α-subunit had pronounced effects on betaglycan binding. In particular, residues Val108 and Tyr210 and, to a lesser extent, Tyr50, Arg109, Thr110, Thr111, Ser112, Ser117, Phe118, and Lys119 are critical for high affinity interactions with betaglycan. These residues form a contiguous epitope on the outer convex surface of the fingers, or knuckle region, of the inhibin α-subunit. It is likely, given its proximity to the binding epitope, that Pro51 is also involved in betaglycan binding; however, mutagenesis of this residue abolished protein expression. The knuckle epitope is a common binding interface for TGFβ superfamily ligands.

Activins, BMPs and growth and differentiation factors bind their type II receptors at this site (8, 12, 31). In addition, follistatin antagonizes the actions of multiple TGFβ ligands by binding to this region and preventing ligand access to their signaling receptors (7, 32, 33).

The current model of betaglycan co-receptor action predicts that betaglycan binds and presents inhibin A to type II receptors (13, 34). Previous studies have localized the inhibin-binding site on betaglycan to the membrane-proximal domain of the molecule (Pro607–Val620) (14). Key residues through this region presumably form hydrophobic and ionic interactions with the corresponding residues on the outer convex surface of the inhibin α-subunit. There are two possible mechanisms that could explain the enhanced affinity of inhibin for type II receptors in the presence of betaglycan: (i) an initial constraint of inhibin at the membrane surface by its high affinity binding receptor (betaglycan) would lead to a decrease in the entropy and, hence, an increase in affinity for the second receptor binding event (ActRII/IB) and (ii) inhibin may facilitate direct interactions between the two receptor molecules. An analysis of structural data on other TGFβ ligands and their receptor complexes supports a model of cooperative oligomeric receptor assembly over direct receptor-receptor interactions (35).

Numerous studies have implicated a role for betaglycan in inhibin A-mediated reproductive functions. Chapman and Woodruff (36) reported that in the rat anterior pituitary, betaglycan was localized to the gonadotrope membrane at the time when inhibin must rapidly reduce FSH to basal levels after the secondary FSH surge. Other studies have shown that betaglycan is expressed in specific cell compartments (i.e. granulosa and thecal cells in the ovary; Leydig and germ cells in the testes) throughout the hypothalamic-pituitary-gonadal axis, consistent with a role for the accessory receptor in inhibin-regulated functions in these cell types (37). However, betaglycan null mutant (BG−/−) mice exhibit embryonic lethality because of heart and liver defects (38), ensuring that the physiological relevance of betaglycan for the reproductive actions of inhibin A have yet to be demonstrated.

To address this issue, we disrupted the betaglycan-binding site on the inhibin α-subunit by the simultaneous substitution to alanine of Thr111, Ser112, and Tyr120. The resultant inhibin variant was unable to suppress activin-induced FSH release by both a mouse pituitary gonadotrope cell line (LβT2) and primary rat pituitary cells in culture. These results support an earlier study that showed that overexpression of betaglycan conferred inhibin responsiveness to cells that are normally refractory to the actions of this hormone (13). Future studies will compare the in vivo biological activities of wild type and mutant inhibin A to conclusively show that betaglycan is essential for inhibin A physiological responses.

Our identification of the betaglycan-binding site on inhibin A also has significant implications for the assembly of the TGFβ receptor complex. The three TGFβ isoforms have been shown to transduce their signals by binding the TGFβ type I and type II receptors, TβRI and TβRII, respectively (39). TGFβ2 differs from TGFβs 1 and 3, however, in that it does not bind TβRII with sufficient affinity to generate responses at biologically relevant concentrations (40). To compensate for this reduced affinity, TGFβ2 forms a complex with betaglycan, also known as the TGFβ type III receptor (41). Because inhibin A and the TGFβ isoforms bind to a common epitope in the membrane-proximal region of betaglycan (14), it is likely that the receptor-binding interface on the ligands is also shared. Thus, we would predict that betaglycan binds to the outer convex surface of the fingers of the TGFβ isoforms. Importantly, this region is adjacent to the TβRII binding interface and has not been implicated in type I receptor interactions (29). Previous studies have suggested that betaglycan presents the TGFβ isoforms to TβRII but that it is then displaced from the ternary complex by TβRI (41, 42). Our model, however, predicts that betaglycan could remain associated with the signaling receptor complex, which in turn could explain how this co-receptor increases cellular responsiveness to TGFβ2.
In conclusion, we have identified and characterized a distinct epitope in the human inhibin α-subunit critical for binding to betaglycan and biological activity. This finding provides new insights into the assembly and activation of receptors for TGFβ superfamily ligands. Utilizing α-subunit mutants, future studies will determine the relative contribution of betaglycan to inhibin B bioactivity and the physiological roles inhibins play in both reproductive and nonreproductive systems.

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