The assembly and secretion of transforming growth factor β superfamily ligands is dependent upon non-covalent interactions between their pro- and mature domains. Despite the importance of this interaction, little is known regarding the underlying regulatory mechanisms. In this study, the binding interface between the pro- and mature domains of the inhibin α-subunit was characterized using in vitro mutagenesis. Three hydrophobic residues near the N terminus of the prodomain (Leu30, Phe37, Leu44) were identified that, when mutated to alanine, disrupted heterodimer assembly and secretion. It is postulated that these residues mediate dimerization by interacting non-covalently with hydrophobic residues (Phe271, Ile280, Pro283, Leu338, and Val340) on the outer convex surface of the mature α-subunit. Homology modeling indicated that these mature residues are located at the interface between two β-sheets of the α-subunit and that their side chains form a hydrophobic packing core. Mutation of these residues likely disturbs the conformation of this region, thereby disrupting non-covalent interactions with the prodomain. A similar hydrophobic interface was identified spanning the pro- and mature domains of the inhibin β-subunit. Mutation of key residues, including Ile62, Leu66, Phe329, and Pro341, across this interface was disruptive for the production of both inhibin A and activin A. In addition, mutation of Ile62 and Leu66 in the βA-propeptide reduced its ability to bind, or inhibit the activity of, activin A. Conservation of the identified hydrophobic motifs in the pro- and mature domains of other transforming growth factor β superfamily ligands suggests that we have identified a common biosynthetic pathway governing dimer assembly.

Inhibin A and B, members of the transforming growth factor β (TGFβ) superfamily, negatively regulate the production and secretion of follicle-stimulating hormone from the anterior pituitary (1, 2), control ovarian follicle development and steroidogenesis (3), and act as tumor suppressors in the gonads (4). Outside the hypothalamic pituitary gonadal axis, inhibins contribute to the endocrine regulation of bone metabolism (5) and play critical roles in adrenal gland growth and function (6, 7). It is recognized that inhibins regulate these processes by inhibiting the stimulatory actions of the structurally related proteins, activins (8). 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. Activins are composed of two β-subunits: βAβA (activin A), βAβB (activin AB), and βBβB (activin B). Inhibin antagonism of activin-related ligands is dependent upon interactions with betaglycan, a cell surface proteoglycan that also acts as a TGFβ2 co-receptor (9). Betaglycan binds inhibin A directly and promotes the formation of a stable high affinity complex involving activin type II receptors (10). Sequestration of type II receptors in this way prevents their interactions with signaling ligands such as activin A or activin B.

Analogous to other members of the TGFβ superfamily, inhibin subunits are synthesized as large precursor molecules. The inhibin α-subunit precursor is divided into three regions by two polypargline cleavage sites (see Fig. 1A): the 43-amino acid proregion; the 171-amino acid αN region; and the 134-amino acid C-terminal (αC) mature region (11). The βA-subunit precursor consists of a 290-amino acid prodomain, separated by a polypargline cleavage sequence from a 116-amino acid C-terminal mature domain (11). During the secretory process, the α- and βA-subunits mature domains fold into a disulfide-linked dimer. The large inhibin precursors are proteolytically cleaved by furin-like proprotein convertases at an RXRR consensus sequence, which separates the prodomains from the mature domains, and the mature inhibin dimers are secreted.

It has been postulated that the prodomains of the α- and βA-subunits are necessary for the correct folding, disulfide bond formation, export, and biological activity of inhibin A (12, 13). Similar regulatory functions have been ascribed to the prodomains of other TGFβ ligands. For example, the prodomain of TGFβ1 (termed latency-associated protein; LAP) represents a functional binding partner for the mature protein (14). The N-terminal region of LAP binds mature TGFβ1 during homodimer assembly and secretion and remains associated following proteolytic cleavage (15–19). LAP binding not only...
blocks TGFβ1 access to its signaling receptors but also sequesters the growth factor to the extracellular matrix (via interactions with latent TGFβ-binding proteins) (20–24). Further proteolysis or conformational changes within LAP are required to release active TGFβ1 (25, 26). The other TGFβ isoforms (TGFβ2 and TGFβ3) and myostatin, and GDF11 also form latent complexes with their prodomains (27, 28).

Recent studies have indicated that complex formation between mature TGFβ ligands and their respective prodomains may be a general phenomenon within the family. Sengel et al. (29) showed that numerous bone morphogenetic proteins, including BMP-2, BMP-4, BMP-7, BMP-10, and GDF5, are secreted as stable complexes consisting of the growth factor domain non-covalently associated with two propeptides. Although prodomain binding for these ligands is not sufficient to confer latency, it is necessary to localize the growth factors to the fibrillarin microfibril within the extracellular matrix (29).

Despite the importance of prodomains in the synthesis and control of TGFβ ligands, little is known about the underlying regulatory mechanisms. In the current study, the binding interface between the pro- and mature domains of the inhibin α-subunit was characterized. Site-directed mutagenesis identified key hydrophobic residues at this interface, which are conserved across the TGFβ superfamily. Based on these investigations, we predict that a common biosynthetic pathway governs the assembly and secretion of TGFβ ligands.

**EXPERIMENTAL PROCEDURES**

**Production of Inhibin Mutants**—Mutations in the pro- and mature regions of the inhibin α- and βA-subunits were introduced using the QuickChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). pCDNA3.1 (Invitrogen) vectors containing either the full-length wild type inhibin α- or βA-subunits served as the templates in these reactions. The mature region of the α-subunit also contained a mutation (N302Q) to ensure that only a 31-kDa inhibin A was produced (30). For each construct, the mutated region was confirmed by DNA sequencing. Wild type and mutant inhibin A and activin A proteins were produced by transient transfection in Chinese hamster ovary (CHO) cells using Lipofectamine (Invitrogen). Briefly, CHO cells were plated at 1 × 10^6 cells/well in a 6-well plate. Wild type or mutant α-subunit DNA (1.6 μg) was combined with βA-subunit DNA (3.3 μg) and Lipofectamine was added according to the manufacturer’s instructions. After a 20-min 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. At 48 h after transfection, the 293T conditioned medium was collected and concentrated 30-fold using Amicon Ultra-15 concentration devices with a 10-kDa regenerated cellulose membrane (Millipore, Billerica, MA). Concentrated protein was diluted in phosphate-buffered saline (pH 7.4) containing protease inhibitors (Complete protease inhibitor mixture tablets, Roche Applied Science, Basel, Switzerland).

**Analysis of the Interaction between the βA-Propeptide and Mature Inhibin A/Activin A**—Conditioned medium from transfected CHO cells expressing wild type and mutant βA-propeptide with a C-terminal FLAG tag was concentrated 50-fold using Nanosep microconcentration devices with a 10-K cut-off (Pall Life Sciences) and resuspended in Laemmli sample buffer. Non-reduced samples were separated by 10% SDS-PAGE and transferred onto ECL Hybond membranes (GE Healthcare). Membranes were blocked in Tris-buffered saline (TBS) with 5% milk. The blocking solution was removed by multiple washes in TBS/bovine serum albumin (3% bovine serum albumin, 0.1% Tween 20), and the membranes were probed with either 125I-inhibin A or 125I-activin A (400,000 cpm/ml) in TBS/bovine serum albumin. After incubation, the tracer was removed by multiple washes with TBS, and the membranes were exposed to film for up to 4 days at −80 °C and developed.

**Immunoprecipitation**—The ability of the βA-propeptide and activin type II receptors (ActRIIA and ActRIIB) to compete for...
binding to mature activin A was assessed by immunoprecipitation. Increasing concentrations of ActRIIA and ActRIIB extracellular domains (25 ng–4 μg; R&D Systems, Minneapolis, MN) were added to samples containing wild type βα-propeptide (400 ng) and activin A (12.5 ng). Samples were immunoprecipitated using a FLAG M2 affinity gel (Sigma-Aldrich) directed against the βα-propeptide. Protein complexes were eluted from the resin using reducing sample buffer and separated by SDS-PAGE. After electrophoresis, samples were transferred onto an ECL Hybond membrane. The activin βα-subunit was detected using the E4 antibody, and βα-propeptide variants were identified using the FLAG M2 antibody.

In Vitro Bioassay—Wild type and mutant (I62A/L66A) βα-propeptides were assessed for their ability to suppress activin A bioactivity in a mouse adrenocortical cell line (7). Briefly, adrenocortical cells were plated in 48-well plates at 114,000 cells/well. After a 24-h incubation, cells were transfected with an activin responsive luciferase reporter construct (pGRAS) using Lipofectamine according to the manufacturer’s protocol (Invitrogen). The cells were washed 24 h after transfection with complete medium and treated with 400 pM activin A and increasing doses of either wild type or mutant βα-propeptides (0.5–30 nM) or activin A (0.1–3 nM). Activin-induced luciferase activity was then determined.

Statistics—Significance (p < 0.05) was determined using one-way t tests for independent groups. In Figs. 1, 3–5, and 7, all error bars shown represent standard deviation.

RESULTS

Hydrophobic Residues in the Prodomain of the Inhibin α-Subunit Regulate Heterodimer Assembly and Secretion—The inhibin α-subunit prodomain comprises a 43-amino acid proregion and a 171-amino acid N-terminal (αN) region. Based on previous studies (15, 17, 33), the proregion (Cys19–Arg61) was identified as the region most likely to be involved in non-covalent interactions with the mature α-subunit. Hydrophobic residues through this region were substituted with alanine using in vitro mutagenesis. In all, a set of 10 variants mutated at 12 different positions was generated (Fig. 1A).

Wild type and mutant proteins were expressed in CHO cells and the conditioned medium and cell lysate were collected. Western blot analysis indicated that conditioned medium from cells transfected with wild type α- and βα-subunits contained both mature (31-kDa) and precursor (65- and 95-kDa) inhibin forms, together with substantial amounts of free α-subunit (50 kDa) (Fig. 1B, lane 1). Mutation of the majority of hydrophobic residues through the proregion had little effect on the amount or the composition of the inhibin forms produced by CHO cells (Fig. 1, B and D). However, three residues (Leu30, Phe37, Leu41) were identified that, when mutated to alanine, resulted in a significant reduction (>80%) in the amount of inhibin A produced and secreted (Fig. 1, B and D). An analysis of the cell lysates from the transfected CHO cells indicated that the decrease in inhibin A production was not due to a loss of α-subunit expression as it was present at similar levels for all mutants tested (Fig. 1C). Rather, the identified point mutations (L30A, F37A, and L41A) appeared to disrupt the dimerization of the α- and βα-subunits, as evidenced by the decrease in dimeric inhibin precursor (Fig. 1C, compare lane 1 with lanes 5, 8, and 10). Together, these results suggest that Leu30, Phe37, and Leu41 are necessary to maintain the inhibin α-subunit in a conformation competent for dimerization with the βα-subunit.

Conservation of the Hydrophobic Motif in the Prodomains of TGFβ Ligands—Sequence alignment (as determined using ClustalW) of the 33 human TGFβ family members indicated that Phe37 and Leu41 of the inhibin α-subunit form part of a
Assembly and Secretion of TGFβ Ligands

Inh α 22LELARELVLAKVRAVFL-DAGPAPAV46
Act A 47VPNSQPHEMRVAYKHKH-NNMLHKKR71
Act B 67LGRVGDHELAVEKRRH-SRLQMRGR81
Act C 36TESQREILDLAKRSTL-DKELTLTQR60
Act E 36PQAERALV-VLACKLDIR-GHELTSR60
TGBA 36MELVRKRRKAEIRCSLA-SKALAS62
TGBB 29MDQFMRRKAEIRCSLA-SKALATSP53
TGBC 37FEGHKRRKAEIRCSLA-SKALATSP56
BMP2 34AASGRSRSEQPSDEL-VSEPLRL58
BMP3 161DLSWTLGKRNLQSSL-GHLWDMA44
BMP4 49RSQSHRRDLRFEATLL-QMFSLRRR70
BMP5 41RRHLHNERGQIR-EL-SGNSRPH44
BMP6 66RRLKQERRRMQK-EBL-SVCLSRLP92
BMP7 48RRLSQERRRMQK-EBL-SVCLSRLP87
BMP8a 34RRLGARREDVQR-EBL-AVLSLPRG57
BMP8b 34RRLGARREDVQR-EBL-AVLSLPRG57
BMP10 41TQGDFNFIL relieved QSMKDFP-ELTLDLDST1
BMP15 29GQSSHIALEEPTLEH-L-EELLESP53
Nodal 42AYMLISYRDLPLRRAI-REGSQAEDY66
GDF1 29PVGPAPAALL-QAGLRDE48
GDF2 51HTPNMLKLEMVFKD-ER-SLNLG75
GDF3 67SRDLCYKELGVRNWI-RFLDPDE91
GDF5 252QYVFDISALE-KDCGILGERLIRK44
GDF6 144EKYLDFWLSMDSKEDLVGAEELRFRQ27
GDF7 99QFSDLVFSNDDAVIEOGAEAVLRR44
GDF8 43RQNTKSSHEAIKQIL-SKLELETA61
GDF9 46WSLLQHIDRAGL-PALFKVLS70
GDF10 155SQRPLPLPPTRQHLF-RISCQSNTA42
GDF11 68QRRSRESLEISGSK-SKALRKEA22
GDF15 158FEDSRFBRLKRYEDDQL-TLDKANQ32
MIS 68SPLRJVGALGAYEQAPL-GAVQRAW94
Leftyl 22LGEGQLLGSL-RQGLKEV40
Lefty2 22LGEGQLLGSL-RQGLKEV40

FIGURE 2. Sequence alignment of prodomains for human TGFβ ligands.
The inhibin α-subunit (Inh α) prodomain was aligned with the prodomains of human TGFβ ligands using ClustalW. The residues are numbered according to the first residue of the signal peptide. The three residues determined in this study to be essential for inhibin dimer formation and secretion (Leu62, Phe77, and Leu41) are highlighted. The identified residues lie within a conserved hydrophobic motif (bottom of alignment). Act, activin.

As activin A levels. As the precursor βA-subunit was present in cell lysates at comparable levels for each of the variants (Fig. 3D), decreased activin and inhibin expression likely occurred because of a defect in folding and/or dimerization. Interestingly, mutation of Val55 of the βA-subunit (corresponding to Leu30 of the α-subunit) had no effect on inhibin A or activin A expression. This residue is upstream of the hydrophobic motif and is less well conserved across the TGFβ family.

The βA-Subunit Prodomain Interacts Directly with Activin A and Inhibin A—The nature of the conformational changes induced by mutation of the βA-subunit residues, Ile62 and Leu66, were then examined. Wild type and mutant (I62A and I62A/L66A) βA-propeptides with a C-terminal FLAG tag were expressed by transient transfection in CHO cells, separated by SDS-PAGE, and transferred to ECL Hybond membranes. A Western blot with the FLAG M2 mAb confirmed that all the βA-propeptides were loaded at equivalent concentrations (Fig. 4A). Probing membranes with 125I-inhibin A or 125I-activin A indicated that both ligands bound strongly to the wild type βA-propeptide (Fig. 4A). In contrast, no detectable binding of inhibin A or activin A was observed with the βA-propeptides carrying mutations in the identified hydrophobic motif (I62A and I62A/L66A). These results indicate that the residues within the hydrophobic motif of the βA prodomain interact directly with the mature βA domain to regulate the assembly and secretion of inhibin A or activin A.

Activin A Binds Propeptide and Type II Receptors through Overlapping Binding Sites—It has recently been demonstrated that the BMP-7 propeptide locks binding of mature BMP-7 to its type II receptor (34). To determine whether the binding site for the βA-propeptide on activin A overlaps with that of activin type II receptors, immunoprecipitation studies were performed. Concentrated culture medium containing βA-propeptide was combined with activin A (R&D Systems) and increasing doses of activin type II receptors (ActRIA and ActRIBB extracellular domains, R&D Systems). Samples were immunoprecipitated using the FLAG M2 affinity resin (Sigma-Aldrich) and analyzed by Western blot using an activin A (E4) antibody. In the absence of activin type II receptors, βA-propeptide formed a complex with activin A (Fig. 4, A and C). However, increasing doses of the activin type II receptor extracellular domains decreased the amount of activin A recovered by immunoprecipitation. This suggested that ActRIBA can displace βA-propeptide from binding to activin A, supporting the concept that these proteins share an overlapping binding epitope on the mature activin A dimer.

The βA-Propeptide Supresses Activin A Bioactivity in Adrenocortical Cells—The adrenocortical cell system was used to determine whether the βA-propeptide was able to block activin biological activity. In this assay, activin A induced a 12-fold increase in luciferase response, which could be blocked with increasing doses of the antagonist, inhibin A (Fig. 4D and data not shown). High doses of wild type βA-propeptide were also able to suppress the activin-induced luciferase response (Fig. 4D), in a manner similar to that previously described for soluble activin type II receptors (35). In contrast, the I62A/L66A βA-propeptide variant was unable to inhibit activin A signaling in the adrenocortical cells. Together, these results suggest that,
if present at high concentrations, the $\beta_A$-propeptide may modulate activin signaling.

Characterization of the Pro- and Mature Domain Binding Interface on the Inhibin $\alpha$-Subunit—The outer convex surface of the “finger” regions of TGF$\beta$ ligands bind type II receptors (36, 37) and, based on competition studies (Fig. 4) (34), likely provide the interface for interactions with propeptides. To identify the binding epitope on inhibin $A$ for the $\alpha$-subunit prodomain, residues through the finger regions of the $\alpha$-subunit were substituted for alanine. In all, a set of 28 variants was generated (38). Western blot analysis of conditioned medium from CHO cells transfected with wild type or mutant $\alpha$-subunit revealed that residues Phe$^{277}$, Ile$^{280}$, Pro$^{283}$, Leu$^{338}$, and Val$^{340}$ in the finger region of the $\alpha$-subunit are necessary for inhibin $A$ production and secretion (Fig. 5B, lanes 2, 4, 6, 7, and 9). The identified residues are distant from the predicted inhibin $\alpha/\beta_A$ dimer interface (see Fig. 8A). An inhibin $A$ ELISA confirmed that mutation of these residues reduced inhibin $A$ production by $>80\%$ (Fig. 5D). The reduction could not be attributed to a loss of expression of the $\alpha$-subunit as it was easily identified in the cell lysates of the transfected CHO cells for all mutants tested (Fig. 5C). As these hydrophobic residues are critical for the correct folding of the $\alpha$-subunit and subsequent dimerization with the $\beta_A$-subunit, it is likely that they constitute the binding epitope for the prodomain residues, Leu$^{30}$, Phe$^{37}$, and Leu$^{41}$. In support, mutation of non-hydrophobic residues (e.g. Tyr$^{282}$ and His$^{339}$) through this region did not affect the formation of inhibin $A$ (Fig. 5, B and D). Moreover, conservative substitutions at positions Leu$^{338}$ (L338M) and Val$^{340}$ (V340L) allowed for inhibin $A$ dimer formation (data not shown).

Conservation of Hydrophobic Residues across the TGF$\beta$ Superfamily—Sequence alignment of the 33 human TGF$\beta$ family members indicated that the five hydrophobic $\alpha$-subunit residues required for the correct folding and dimerization of inhibin $A$ are highly conserved (Fig. 6). Pro$^{283}$ (inhibin $\alpha$-subunit numbering) is invariant across the family, whereas Phe$^{277}$ and Ile$^{280}$ are present in most family members. At other positions in the hydrophobic motif (Leu$^{338}$ and Val$^{340}$), conservative amino acid substitutions are noted. The corresponding hydrophobic residues in the mature region of the $\beta_A$-subunit were substituted for alanine (Fig. 7A). As anticipated, in cells co-transfected with wild type $\alpha$-subunit, these $\beta_A$-subunit point mutations (F329A, I338A, P341A, M398A, and M400A) were disruptive for activin $A$ expression (Fig. 7). Interestingly, inhibin $A$ expression was only significantly reduced in the P341A and M400A $\beta_A$-subunit variants (Fig. 7E), suggesting that it is less dependent on $\beta_A$-subunit conformation.

The effects of these amino acid substitutions on inhibin $A$ and activin $A$ production, culture medium from CHO cells transfected with either wild type (lane 1) or mutant $\beta_A$-subunit (lanes 2–4), in combination with the $\alpha$-subunit, were analyzed by Western blot. Blots were probed with the E4 mAb, specific for the inhibin/activin mature $\beta_A$ domain (8) and the R1 mAb specific for the inhibin $\alpha C$ (mature) domain (G). D. Western blot analysis of the cell lysates of CHO cells transfected with either wild type (lane 1) or mutant $\beta_A$-subunit cDNAs (lanes 2–4) is shown. The 31-kDa inhibin $A$ dimer, 24-kDa activin $A$ dimer, 54-kDa free $\beta_A$-subunit, and higher molecular mass precursor forms of inhibin and activin are noted. The effect of $\beta_A$-subunit prodomain mutations on activin $A$ (E) and inhibin $A$ (F) expression in CHO culture medium was also determined by ELISA (* $p < 0.05$). WT, wild type.
Molecular Modeling of the Inhibin A Dimer—Previously, we constructed a homology model of inhibin A (38) based on the activin A, BMP-3, and BMP-6 structures (7, 12, 21). Mapping of the inhibin α- and βₐ-subunit residues mutated in this study onto the modeled structure of inhibin A is shown in Fig. 8A. The α-subunit residues Phe²⁷₁, Ile²⁸₀, Pro²⁸³, Leu³³⁸, and Val³⁴⁰ are located at the interface between two β-sheets, and the side chains of these residues form a hydrophobic packing core. Disruption to this region could affect the folding or stability of mature inhibin A, which may explain the low levels of mutant proteins detected in CHO cell conditioned medium (Fig. 5).

DISCUSSION

The prodomains of TGFβ family members play an important role in the biosynthesis of these ligands. As a consequence, naturally occurring mutations within the prodomains of TGFβ ligands are often associated with disease pathologies. Mutations in the proregions of GDF9 and BMP-15 have been identified in patients diagnosed with premature ovarian failure (39–41). Patients presenting with Camurati-Engelmann disease, which is characterized by alterations in bone density resulting in severe bone pain, have been found to carry mutations in the TGFβ1 prodomain (42–44). Mutations are also prevalent in the proregion of Müllerian-inhibiting substance (MIS, or anti-Müllerian hormone), which result in persistent Müllerian duct syndrome, an autosomal recessive intersex disorder (45). A greater understanding of the mechanisms by which the prodomains assist the formation and/or functions of TGFβ ligands would aid the development of future treatments for these conditions.

In this study, utilizing a site-directed mutagenesis approach, we have provided a structural basis for understanding the critical role that prodomains play in facilitating the assembly and secretion of inhibin A and related TGFβ ligands. Mutagenesis of residues in the N-terminal portion of the βₐ-subunit prodomain had pronounced effects on inhibin A production. In particular, residues Phe³⁷ and Leu⁴₁ and, to a lesser extent, Leu³⁰ are critical for maintaining the βₐ-subunit in a conformation competent for dimerization with the βₐ-subunit. Crystal structures are not available for the propeptides of any TGFβ ligands; however, molecular modeling has provided insights into the structural basis for propeptidemediated dimerization. The βₐ-subunit dimerization interface is critical for the assembly of inhibin A and related TGFβ ligands.

FIGURE 4. Analysis of the interaction between the βₐ-propeptide and mature inhibin and activin A dimers. A, ligand blot analysis of wild type (WT) and mutant βₐ-propeptide binding to ¹²⁵I-inhibin A and ¹²⁵I-activin A dimers. Wild type and mutant βₐ-propeptide (with C-terminal FLAG tag) were loaded at equivalent concentrations (as determined by Western blotting with the FLAG M2 mAb, top panel) onto SDS-PAGE and transferred to an ECL Hybond membrane. Membranes were probed with either ¹²⁵I-activin A (middle panel) or ¹²⁵I-inhibin A (bottom panel). B and C, the ability of the activin type II receptors (ActRIIA and ActRIIB) to compete with the βₐ-propeptide (proβA) for binding to mature activin A was assessed by immunoprecipitation. Increasing concentrations of ActRIIA (B) and ActRIIB (C) extracellular domains (ECD) (25 ng–4 μg; R&D Systems) were added to samples containing wild type proβA-FLAG (400 ng) and activin A (12.5 ng). Samples were immunoprecipitated (IP) using FLAG M2 affinity resin and detected by immunoblot (IB) using the activin subunit mAb (E4). To ensure that equal amounts of activin and proβA-propeptides were present in each of the samples, immunoblots using the FLAG M2 and E4 antibodies were also performed prior to immunoprecipitation. D, in vitro bioassay to assess the ability of wild type and mutant βₐ-propeptides to block activin signaling. Adrenocortical cells were transfected with an activin responsive luciferase reporter and treated with 400 pm activin A (Act A) and increasing doses of either wild type or mutant βₐ-propeptides (0.5–30 nM) (*p < 0.05).
however, the three identified hydrophobic residues are predicted to lie within an α-helix (determined using NNpredict, data not shown) and likely provide a binding surface for non-covalent interactions with the mature α-subunit. Sequence alignment of the proregions of the 33 human TGFβ family members revealed that α-subunit residues Phe37 and Leu41 lie within a conserved hydrophobic motif (37Hyd-Hyd-X-Hyd-X-Hyd43). The conservation of hydrophobicity suggests that this region serves a common role in governing the assembly and secretion of TGFβ ligands. Several pieces of evidence support this concept. (i) Mutation of the corresponding hydrophobic residues in the βA-subunit (Ile62 and Leu66) disrupted both inhibin A and activin A dimerization (Fig. 3); (ii) deletion of residues within the identified hydrophobic motif of TGFβ1 blocked the association between the pro- and mature domains and inhibited the secretion of mature TGFβ1 (17); (iii) the TGFβ1 physiological activator, thrombospondin 1, binds to residues (30LSKL37) within the identified motif (46); and (iv) Jiang et al. (33) have mapped the inhibitory domain of the myostatin propeptide to residues 42–115, which encompasses the hydrophobic motif identified in the inhibin subunits.

Recent studies on TGFβ1, myostatin, and BMPs have indicated that after cleavage, propeptides retain the capacity to interact non-covalently with their respective dimeric growth factors (27, 28, 34). Immunoprecipitation and ligand blot studies demonstrated that this was also the case for the inhibin and activin isoforms. Isolated βA-propeptide was able to bind directly to inhibin A and activin A. Importantly, mutation of Ile62 or Leu66 within the βA-propeptide completely abrogated interaction with the mature ligands, confirming that these residues are central to the non-covalent interactions between the pro- and mature domains. For some family members, including the TGFβ isoforms, myostatin and GDF11, high affinity interactions with isolated propeptides is sufficient to confer latency (17, 27, 28). This fact has been utilized successfully for the in vivo inhibition of myostatin and, hence, muscle growth. (27–
For other ligands (e.g., BMP-7 and BMP-9), propeptides bind with lower affinity and are unable to suppress biological activity (34, 50). In the current study, bioactivity assays indicated that the inhibin A-propeptide could inhibit activin activity, but only at high concentrations. Thus, the affinity of the
Assembly and Secretion of TGFβ Ligands

The βA-propeptide reduced activin signaling because at high concentrations, it was capable of displacing activin A from binding to type II receptors (ActRII/IIB). In similar experiments, Sengle et al. (34) recently demonstrated that the BMP-7 propeptide competes with BMPRII for binding to the mature ligand. In addition, the sequence K94RKP97 in the receptor-binding region of mature TGFβ1 has been implicated in binding LAP (17). Together, these studies suggest that the binding epitopes for prodomains and type II receptors overlap on TGFβ ligands (i.e. both bind to the outer convex surface of the finger regions). Using this information as a guide, we set out to identify the residues in the mature domains of the inhibin A subunits that form non-covalent interactions with their respective prodomains. After extensive mutagenesis, it was found that alanine substitution of a number of hydrophobic residues (Phe²⁷¹, Ile²⁸⁰, Pro²⁸³, Leu³³⁸, and Val³⁴⁰) in the finger regions of the α-subunit were disruptive for the formation of inhibin A dimers in vitro. The identified residues are located at the interface between two β-sheets of the α-subunit, and the side chains of these residues form a hydrophobic pocket (Fig. 8). It is likely that mutation of some of these residues (Phe²⁷¹, Ile²⁸⁰, and Leu³³⁸) perturbs the local conformation of the mature domain, thereby hindering the ability of the prodomain to bind. However, for the surface-exposed residues, Pro²⁸³ and Val³⁴⁰, mutations may directly disrupt hydrophobic interactions with the prodomain. In support, mutation of these residues has previously been shown to disrupt inhibin A binding to its co-receptor, betaglycan (38).

Mutations of the corresponding residues within the βA-subunit were also found to abrogate the expression of activin A. Interestingly, these mutations were significantly less disruptive for inhibin A expression, suggesting that the α-subunit drives inhibin production. Sequence alignment of the mature domains of the 33 human TGFβ ligands revealed that the identified hydrophobic residues are highly conserved across the family, suggesting that this region plays a common structural role in the formation of these ligands. In support, a naturally occurring mutation (V⁴⁷⁷A) in the mature region of MIS, which corresponds to Ile²⁸⁰ in the inhibin α-subunit, has been identified in patients with persistent Müllerian duct syndrome. The V⁴⁷⁷A mutation in MIS disrupts protein production, reducing the circulating levels of MIS by 90% (45).

In conclusion, our data indicate that a common biosynthetic pathway governs the production and secretion of TGFβ ligands. In this model (Fig. 8B), hydrophobic residues within the N-terminal portion of the prodomain and the finger regions of the mature domain interact non-covalently, maintaining the molecule in a conformation competent for dimerization (the actual dimerization interface for the two monomers, close to the cysteine knot motif, is well removed from this prodomain-binding site). Dimeric precursors are cleaved by furin-like proconvertases at RXXR sites that separate the propeptides from the mature domains. The dimeric, mature ligands are then secreted from the cell non-covalently associated with their respective prodomains. For inhibin A, the α- and βA-propeptides are readily displaced by betaglycan and activin type II receptors, respectively, ensuring that this hormone is secreted in an active state. Other ligands (e.g. TGFβ1 and myostatin) have higher affinities for their prodomains and are secreted in a latent form.

REFERENCES

1. Robertson, D. M., Giacometti, M. S., and de Kretser, D. M. (1986) Mol. Cell. Endocrinol. 46, 29–36
2. Woodruff, T. K., Krummen, L. A., Lyon, R. J., Stocks, D. L., and Mather, J. P. (1993) Endocrinology 132, 2332–2341
3. Woodruff, T. K., Lyon, R. J., Hansen, S. E., Rice, G. C., and Mather, J. P. (1990) Endocrinology 127, 3196–3205
4. Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J., and Bradley, A. (1992) Nature 360, 313–319
5. Perrien, D. S., Akel, N. S., Edwards, P. K., Carver, A. A., Bendre, M. S., Swain, F. L., Skinner, R. A., Hogue, W. R., Nicks, K. M., Pierson, T. M., Suva, L. J., and Gaddy, D. (2007) Endocrinology 148, 1654–1665
6. Farnsworth, P. G., Harrison, C. A., Leembruggen, P., Chan, K. L., Stanton, P. G., Ooi, G. T., Rahman, N. A., Huhtaniemi, I. T., Findlay, J. K., and Robertson, D. M. (2001) Mol. Cell. Endocrinol. 180, 63–71
7. Farnsworth, P. G., Wang, Y., Leembruggen, P., Ooi, G. T., Harrison, C., Robertson, D. M., and Findlay, J. K. (2006) Endocrinology 147, 451–465
8. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986) Nature 321, 776–779
9. Lewis, K. A., Gray, P. C., Blount, A. L., MacConell, L. A., Wiater, E., Bilezikjian, L. M., and Vale, W. (2000) Nature 404, 411–414
10. Wiater, E., and Vale, W. (2003) J. Biol. Chem. 278, 7934–7941
11. Mason, A. J., Farnsworth, P. G., and Sullivan, J. (1996) Mol. Endocrinol. 10, 1055–1065
12. Gray, A. M., and Mason, A. J. (1990) Science 247, 1328–1330
13. Russell, D. L., Doughton, B. W., Tsonis, C. G., and Findlay, J. K. (1994) J. Reprod. Fertil. 100, 115–122
14. Gentry, L. E., Webb, N. R., Lim, G. J., Brunner, A. M., Ranchalis, J. E., Twardzik, D. R., Lioubin, M. N., Marquardt, H., and Purchio, A. F. (1987) Mol. Cell. Biol. 7, 3418–3427
15. Sha, X., Yang, L., and Gentry, E. L. (1991) J. Cell Biol. 114, 827–839
16. De Crescenzo, G., Grothe, S., Zwaagstra, J., Tsang, M., and O’Connor-McCourt, M. D. (2001) J. Biol. Chem. 276, 29632–29643
17. Young, G. D., and Murphy-Ullrich, J. E. (2004) J. Biol. Chem. 279, 38032–38039
18. Bottinger, E. P., Factor, V. M., Tsang, M. L., Weatherbee, J. A., Kopp, J. B., Qian, S. W., Wakefield, L. M., Roberts, A. B., Thorgerisson, S. S., and Sporn, M. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5877–5882
19. McMahon, G. A., Dignam, J. D., and Gentry, L. E. (1996) Biochem. J. 313, 343–351
20. Taijale, J., Saharinen, J., Hedman, K., and Keski-Oja, J. (1996) J. Histochem. Cytochem. 44, 875–889
21. Maeda, S., Dean, D. D., Gomez, R., Schwartz, Z., and Boyan, B. D. (2002) Calcif. Tissue Int 70, 54–65
22. Hyttiainen, M., Penttinen, C., and Keski-Oja, J. (2004) CRC Crit. Rev. Clin. Lab. Sci. 41, 233–264
23. Saika, S., Yamanaka, O., Baba, Y., Kawashima, Y., Shirai, K., Miyamoto, T., Okada, Y., Ohnishi, Y., and Ooshima, A. (2001) Graefe’s Arch. Clin. Exp. Ophtalmol. 239, 234–241
24. Taijale, J., Miyazono, K., Heldin, C. H., and Keski-Oja, J. (1994) J. Cell Biol. 124, 171–181
25. Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) Mol. Cell. Biol. 8, 4162–4168
26. Munger, J. S., Harpel, J. G., Gleizes, P. E., Mazzieri, R., Nicks, L., and Rifkin, D. B. (1997) Kidney Int. 51, 1376–1382
27. Ge, G., Hopkins, D. R., Ho, W. B., and Greenspan, D. S. (2005) Mol. Cell. Biol. 25, 5846–5858
28. Thies, R. S., Chen, T., Davies, M. V., Tomkinson, K. N., Pearson, A. A., Shakey, Q. A., and Wolfman, N. M. (2001) Growth Factors 18, 251–259
29. Sengle, G., Charbonneau, N. L., Ono, R. N., Sasaki, T., Alvarez, J., Keene, D. R., Bachinger, H. P., and Sakai, L. Y. (2008) J. Biol. Chem. 283, 9319
Makanji, Y., Harrison, C. A., Stanton, P. G., Krishna, R., and Robertson, D. M. (2007) *Endocrinology* **148**, 2309–2316

Robertson, D. M., Stephenson, T., Pruyers, E., Burger, H. G., McCloud, P., Tsigos, A., Groom, N., Manners, P., McNeilage, J., Jobling, T., and Healy, D. (2002) *Mol. Cell. Endocrinol.* **191**, 97–103

Harrison, C. A., Chan, K. L., and Robertson, D. M. (2006) *Endocrinology* **147**, 2744–2753

Jiang, M. S., Liang, L. F., Wang, S., Ratovitski, T., Holmstrom, J., Barker, C., and Stotish, R. (2004) *Biochem. Biophys. Res. Commun.* **315**, 525–531

Sengle, G., Ono, R. N., Lyons, K. M., Bachinger, H. P., and Sakai, L. Y. (2008) *J. Mol. Biol.* **381**, 1025–1039

del Re, E., Sidis, Y., Fabricio, D. A., Lin, H. Y., and Schneyer, A. (2004) *J. Biol. Chem.* **279**, 53126–53135

Thompson, T. B., Woodruff, T. K., and Jardetzky, T. S. (2003) *EMBO J.* **22**, 1555–1566

Schreuder, H., Liesum, A., Pohl, J., Kruse, M., and Koyama, M. (2005) *Biochem. Biophys. Res. Commun.* **329**, 1076–1086

Makanji, Y., Walton, K. L., Wilce, M. C., Chan, K. L., Robertson, D. M., and Harrison, C. A. (2008) *J. Biol. Chem.* **283**, 16743–16751

Dixit, H., Rao, I. K., Padmalatha, V. V., Kanakavalli, M., Deenadayal, M., Gupta, N., Chakrabarty, B., and Singh, L. (2006) *Hum. Genet.* **119**, 408–415

Di Pasquale, E., Rossetti, R., Marozzi, A., Bodega, B., Borgato, S., Cavallo, L., Einaudi, S., Radetti, G., Russo, G., Sacco, M., Wasiennska, M., Cole, T., Beck-Peccoz, P., Nelson, L. M., and Persani, L. (2006) *J. Clin. Endocrinol. Metab.* **91**, 1976–1979

Di Pasquale, E., Beck-Peccoz, P., and Persani, L. (2004) *Am. J. Hum. Genet.* **75**, 106–111

Campos-Xavier, B., Saraiva, J. M., Savarirayan, R., Verloes, A., Feingold, J., Faire, L., Munnich, A., Le Merrer, M., and Cormier-Daire, V. (2001) *Hum. Genet* **109**, 653–658

Janssens, K., Vanhoenacker, F., Bonduelle, M., Verbruggen, L., Van Maldergem, L., Ralston, S., Guanalens, N., Migone, N., Wientroub, S., Divizia, M. T., Bergmann, C., Bennett, C., Simsek, S., Melancon, S., Cundy, T., and Van Hul, W. (2006) *J. Med. Genet.* **43**, 1–11

Wu, S., Liang, S., Yan, Y., Wang, Y., Li, F., Deng, Y., Huang, W., Yuan, W., Luo, N., Zhu, C., Wang, Y., Li, Y., Liu, M., and Wu, X. (2007) *Bone* **40**, 1630–1634

Belville, C., Van Vlijmen, H., Ehrenfels, C., Pepinsky, B., Rezaie, A. R., Picard, J. Y., Josso, N., di Clemente, N., and Cate, R. L. (2004) *Mol. Endocrinol.* **18**, 708–721

Young, G. D., and Murphy-Ullrich, J. E. (2004) *J. Biol. Chem.* **279**, 47633–47642

Yang, J., Ratovitski, T., Brady, J. P., Solomon, M. B., Wells, K. D., and Wall, R. J. (2001) *Mol. Reprod. Dev.* **60**, 351–361

Sunada, Y. (2006) *Rinsho Shinkeigaku* **46**, 942–944

Bogdanovich, S., Perkins, K. J., Krag, T. O., Whittemore, L. A., and Khurana, T. S. (2005) *FASEB J.* **19**, 543–549

Brown, M. A., Zhao, Q., Baker, K. A., Naik, C., Chen, C., Pukac, L., Singh, M., Tsareva, T., Parice, Y., Mahoney, A., Roschke, V., Sanyal, I., and Choe, S. (2005) *J. Biol. Chem.* **280**, 25111–25118