Identification of an Inhibin Receptor in Gonadal Tumors from Inhibin α-Subunit Knockout Mice*

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Inhibins and activins are dimeric proteins that are functional antagonists and are structurally related to the transforming growth factor-β (TGFβ) family of growth and differentiation factors. Receptors for activin and TGFβ have been identified as dimers of serine-threonine kinase subunits that regulate cytoplasmic proteins known as Smads. Despite major advances in our understanding of activin and TGFβ receptors and signaling pathways, little is known about inhibin receptors or the mechanism by which this molecule provides a functionally antagonistic signal to activin. Studies described in this paper indicate that an independent inhibin receptor exists. Numerous tissues were examined for inhibin-specific binding sites, including the developing embryo, in which the spinal ganglion and trigeminal ganglion-bound iodinated inhibin A. Sex cord stromal tumors, derived from male and female inhibin α-subunit-deficient mice, were also identified as a source of inhibin receptor. Abundant inhibin and few activin binding sites were identified in tumor tissue sections by in situ ligand binding using iodinated recombinant human inhibin A and 125I-labeled recombinant human inhibin A. Tumor cell binding was specific for each ligand (competed by excess unlabeled homologous ligand and not competed by heterologous ligand). Based on these results and the relative abundance and homogeneity of tumor tissues versus the embryonic ganglion, tumor tissues were homogenized, membrane proteins were purified, and putative inhibin receptors were isolated using an inhibin affinity column. Four proteins were eluted from the column that bind iodinated inhibin but not iodinated activin. These data suggest that inhibin-specific membrane-associated proteins (receptors) exist.

In 1932, McCullagh (1) proposed that the gonads produce a nonsteroidal factor that regulates pituitary function. The factor was called inhibin and was finally isolated from bovine, ovine, and porcine follicular fluid in 1985 (2). Concurrent purification of FSH-stimulating factors or the mechanism by which this molecule provides a functionally antagonistic signal to activin. Studies described in this paper indicate that an independent inhibin receptor exists. Numerous tissues were examined for inhibin-specific binding sites, including the developing embryo, in which the spinal ganglion and trigeminal ganglion-bound iodinated inhibin A. Sex cord stromal tumors, derived from male and female inhibin α-subunit-deficient mice, were also identified as a source of inhibin receptor. Abundant inhibin and few activin binding sites were identified in tumor tissue sections by in situ ligand binding using iodinated recombinant human inhibin A and 125I-labeled recombinant human inhibin A. Tumor cell binding was specific for each ligand (competed by excess unlabeled homologous ligand and not competed by heterologous ligand). Based on these results and the relative abundance and homogeneity of tumor tissues versus the embryonic ganglion, tumor tissues were homogenized, membrane proteins were purified, and putative inhibin receptors were isolated using an inhibin affinity column. Four proteins were eluted from the column that bind iodinated inhibin but not iodinated activin. These data suggest that inhibin-specific membrane-associated proteins (receptors) exist.

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1 The abbreviations used are: FSH, follicle-stimulating hormone; TGF, transforming growth factor; rh, recombinant human; PAGE, polyacrylamide gel electrophoresis.
negative interaction with receptor subunits. Receptors for many of the individual ligands have been identified, and they have structural characteristics similar to those described for the activin receptor. Inhibin \( \alpha \)-subunit is distinct within the TGF\( \beta \) superfamily because it is capable of heterodimeric assembly (with activin \( \beta \)-subunit) and is not able to homodimerize. The inhibin \( \alpha \)-subunit is one of four proteins distantly related to the core ligands (activin/TGF\( \beta \))/bone morphogenic protein. Other ligands with distant homology include Mullerian inhibiting substance (the receptor of which is analogous to the TGF\( \beta \) structure), growth differentiation factor-9 (the receptor of which includes a glycosylphosphatidylinositol-anchored binding protein that presents the ligand to a tyrosine kinase receptor) (18–20).

An alternative hypothesis is that a separate inhibin receptor or inhibin accessory protein exists that mediates an inhibin-specific signal. Supporting the hypothesis that an independent inhibin receptor exists, inhibin-specific binding sites have been identified in ovaries and testes from mice deficient in the inhibin \( \alpha \)-subunit (21–23). The most compelling evidence indicative of an inhibin receptor or cell surface ancillary binding protein is the identification of an inhibin-specific protein complex in a hematopoietic cell line (24). Taken together, these data indicate that inhibin activity involves an inhibin-specific receptor. Therefore, studies were initiated to isolate the inhibin receptor.

Mice that are genetically deficient in the inhibin \( \alpha \)-subunit are deficient in inhibin A and inhibin B and overexpress activin A and activin B (24, 25). These inhibin knockout mice are normal during embryonic and early postnatal development. However, microscopic focal gonadal tumors of the mixed or incompletely differentiated sex cord stromal (granulosa/Sertoli cell) type develop in mice as early as 4 weeks of age. Eventu-

ally, 100% of these mice will have either unilateral or bilateral tumors and die of a cancer cachexia-like syndrome due to the activin secreted from the tumors (26). Upon investigation, these tumors were found to bind inhibin specifically, and data are presented identifying tumor-derived inhibin receptor proteins.

**EXPERIMENTAL PROCEDURES**

**Recombinant Ligands**—Recombinant human (rh)-inhibin A and rh-activin A were obtained from Genentech, Inc. (South San Francisco, CA). The ligands were formulated in a buffer of 0.15 M NaCl and 0.05 M Tris, pH 7.4. Recombinant human follistatin (288aa) was obtained from the National Hormone and Pituitary Distribution Program (NIDDK, National Institutes of Health).

**Iodination of Ligands**—rh-activin A and rh-inhibin A were iodinated by a modified lactoperoxidase method. Briefly, 5 \( \mu \)g of ligand was diluted in 0.4 M sodium acetate, pH 5.6, and 0.5 nmol of Na\(^{125}\)I (0.5 \( \mu \)Ci/\( \mu \)g) on ice at 0°C. The ligands were incubated at ambient temperature with intermittent vortexing for 5 min. The reaction was quenched with 450 \( \mu \)l of phosphate-buffered saline + 0.05% Tween 20 + 0.5% bovine serum albumin (Intergene, Purchase, NY). A 10-\( \mu \)l aliquot of the precolumn fraction was removed for trichloroacetic acid precipitation. Free iodine was removed using Sephadex G-10 column chromatography (PD-10, Pharmacia Biotech Inc.). The specific activity of the ligands was approximately 100 \( \mu \)Ci/\( \mu \)g (range, 80–102 \( \mu \)Ci/\( \mu \)g). To verify that the iodinated inhibin and activin used in these studies were active, ligands were assayed for bioactivity by perifusion of dispersed rat pituitary cells and quantitation of FSH mRNA (27). Both ligands retained full biological activity (data not shown). Moreover, activin was able to bind type RII receptor expressed in a rat pituitary cell line (ovT3), indicating that this ligand would be able to bind its receptor in the eluate if it existed (data not shown).

**In Situ Ligand Binding**—In situ ligand binding was performed as described previously using rat embryos collected on E13, E15, and E17 or control ovaries and gonadal tumors collected from inhibin \( \alpha \)-subunit knockout mice (22, 28). Briefly, 12-\( \mu \)m cryocut tissue sections were incubated for 3 h at room temperature in blocking buffer: Dulbecco’s modified Eagle’s medium:F-12 (1:1), 20 mM HEPES, 0.05% cytochrome C, 0.3% bovine serum albumin, 0.01% phenylmethylsulfonyl fluoride, 0.01% bacitracin, 0.4 \( \mu \)g/ml leupeptin. Slides were then incubated at room temperature overnight in the same buffer containing 40 pmol \(^{125}\)I-rh-inhibin A, 40 pmol \(^{125}\)I-rh-activin A or in the presence of 40 nmol of excess homologous ligand (to define nonspecific background) or heterologous ligand (to define low affinity binding to heterologous ligands). The slides were washed in phosphate-buffered saline (two times for 10 min each); fixed in 3.7% formalin, 2% glutaraldehyde (10 min); rinsed in...
**RESULTS**

The successful identification of activin/TGFβ/BMP/MIS receptor subunits has proceeded based on degenerate oligonucleotide priming from the conserved serine-threonine kinase domain of the activin receptor subunit. Studies in our laboratory were initiated to identify a subunit receptor that bound inhibin and not activin using degenerate oligonucleotides against the conserved serine-threonine kinase domain in pituitary and inhibin α-subunit tumor tissue. No independent receptor isoform was identified using this approach (data not shown). Second, an expression library generated from rat ovaries was screened for inhibin-binding proteins. No inhibin-specific binding proteins were identified using this methodology (data not shown).

Ligand binding studies in ovary (22), testis (23), and the developing embryo (Fig. 1) indicate that separate inhibin and activin binding sites exist in specific cellular groups. In the embryo, inhibin-specific binding sites are detected in the trigeminal ganglion and spinal ganglion. Because activin receptors and follistatin are also present near or coincident with the inhibin binding sites in ovary, testis, and the embryo, no attempt was made to purify the inhibin receptor from these source tissues (22, 23, 28). These studies indicate, however, that several tissues or cellular groups have inhibin binding sites that are distinct from activin binding sites.

Based on our *in situ* ligand binding studies, we hypothesized that gonadal tumor tissues arising from the genetic deletion of the inhibin α-subunit in mice would be a potential source of inhibin receptor. Gonadal tumors (both ovarian and testicular) were collected from adult inhibin α-subunit-deficient mice and embedded immediately on dry ice. Tumor tissues were processed to examine inhibin or activin binding sites using *in situ* ligand binding (Fig. 2). The tumors had a mixed sex cord stromal phenotype, and all cells bound labeled ligands (Fig. 2, B–D). The tumors were analyzed by nonreducing SDS-PAGE. The gels were dried and subjected to autoradiography.

**Fig. 2. In situ ligand binding of 125I-rh-inhibin A and 125I-rh-activin A to normal mouse ovary sections (A and E) and mouse ovarian tumor sections (B–D and F–H). The top row (A–D) are autoradiographic film images of tissues incubated with 125I-rh-inhibin A. The bottom row (E–H) are adjacent tissue sections incubated with 125I-rh-activin A. The concentration of labeled ligand in each case was 40 pM. The concentration of unlabeled ligand was 40 nM. Twenty-four gonadal tumors were evaluated in three separate experiments, and the results presented are representative. Low levels of 125I-rh-inhibin A binding were noted in control ovary sections (A), which is identical to that shown previously in the rat. High levels of 125I-rh-inhibin A binding to all cells of the gonadal tumors was noted (B). The 125I-rh-inhibin A binding was competed by unlabeled rh-inhibin A (C) and not competed by unlabeled rh-activin A (D). In contrast, the control ovarian tissue sections had easily detectable 125I-rh-activin A binding to follicle structure (E) in a pattern identical to that demonstrated previously in the rat. The tumor tissue had low 125I-rh-activin A binding (F), which was competed by activin (G) but not inhibin (H). This low level activin binding may represent interaction of the 125I-rh-activin A probe with follistatin or activin type II receptor subunit.**

**Fig. 3. Analysis of ovarian and testicular tumor homogenates for inhibin binding (receptor) proteins. Ovarian and testicular tumor homogenates were prepared, and membrane proteins were solubilized. Homogenates were incubated with 125I-rh-inhibin A, cross-linked, and analyzed by nonreducing SDS-PAGE. Specificity of the 125I-rh-inhibin A binding was determined by competition experiments in which 100-fold unlabeled inhibin was co-incubated with 125I-rh-inhibin A and protein homogenates. Both ovarian and testicular tumors contained proteins that bound 125I-rh-inhibin A, and the ability of 125I-rh-inhibin A to bind these proteins was competed for partially (ovarian) or entirely (testicular) by unlabeled inhibin A, suggesting that the proteins cross-linked to the labeled ligands did so in a specific manner (Precolumn fractions). Ovarian or testicular homogenates were passed over an inhibin affinity column, and proteins eluted from the column were analyzed by cross-linking study (Postcolumn fractions). Proteins of similar apparent molecular weights were identified in ovarian and testicular homogenates, and these proteins corresponded to the proteins that were specifically competed by unlabeled inhibin A. The arrow indicates the position of 125I-rh-inhibin A. The lettered bands are proteins that correspond to proteins isolated in all purifications (for example, see Fig. 4, proteins a, b, and c).**
with heterologous labeled ligand, indicating that the tissues have distinct inhibin and activin binding sites (Fig. 2, D and H).

Sections of ovaries obtained from wild-type littermate animals had low level inhibin binding to antral granulosa cells (Fig. 2A) and higher levels of activin A binding to ovarian follicles (Fig. 2E). The pattern and intensity of inhibin and activin binding in control mouse ovaries is identical to the binding pattern of the two ligands previously described in normal rat ovary (22).

Because the tumor tissues appeared to be enriched for an inhibin binding moiety and had little activin binding (which we predicted would represent binding to follistatin, a cytoplasmic protein), ovarian and testicular tumor tissues were homogenized, and membrane proteins were isolated from multiple tumors. Protein was incubated with iodinated inhibin, cross-linked with diisuccinimidyl suberate, and analyzed under non-reducing conditions on denaturing SDS-PAGE (Fig. 3). In addition, iodinated inhibin formed complexes with proteins in both ovarian and testicular tumor extracts. Incubation of membrane extracts with 100-fold excess unlabeled rh-inhibin A reduced the inhibin binding in ovarian tumor extracts and competed efficiently for the binding in testicular tumor homogenates. Ovarian and testicular tumor homogenates were passed over an inhibin affinity column, resulting in an enrichment of proteins that bind inhibin. Ovarian and testicular tumor homogenates were combined from this experiment forward.

To examine whether the proteins that could be isolated would bind specifically to inhibin, solubilized membrane protein from ovarian and testicular tumors was passed over the inhibin affinity column twice, the column was washed, and the protein that bound the immobilized inhibin was eluted using a low pH buffer. Fractions representing the eluted protein peak were neutralized with Tris, and aliquots were incubated with iodinated inhibin and iodinated activin. The proteins were cross-linked with diisuccinimidyl suberate and analyzed by SDS-PAGE (Fig. 4). When incubated with fractions representing the protein peak eluted from the inhibin affinity column, $^{125}$I-rh-inhibin A was specifically shifted upward in the chromatogram, whereas $^{125}$I-rh-activin A was not (Fig. 4, A and B, lanes 5–9). Four specific $^{125}$I-rh-inhibin A complexes corresponding to sizes of 130, 116, 86, and 72 kDa were identified (Fig. 4A, lane 7, a, b, c, and d, respectively). These proteins were identified in four independent experiments. However, attempts to microsequence the proteins were unsuccessful due to blocked N termini or lack of sufficient material. Internal sequence analysis was attempted in the cases of blocked N termini; however, no sequence data were generated due to the small amount of protein recovered in the purifications.

To confirm that the proteins were receptors and did not include follistatin, two experiments were conducted. First, in-
Inhibin Receptors in Gonadal Tumors

Inhibin is an gonad-derived dimeric protein hormone. The principle biological activity of inhibin is to suppress pituitary FSH secretion in a classic endocrine fashion (2, 29, 30). It may also participate in ovarian follicle development and oocyte maturation (31–33). Closely related to inhibin (αβ) is activin (ββ). Activin stimulates pituitary FSH synthesis and secretion in a dose-dependent manner and causes follicle atresia; however, it is synergistic with inhibin in stimulating oocyte maturation (2, 21, 31–33). In addition to its role in ovarian and pituitary function, activin is known to regulate erythroid differentiation (34), promote neuronal survival (35), and regulate mesoderm development in Xenopus and mouse embryos (36, 37). Activin regulates these functions through a family of receptor kinases (38–40). One subunit, the type RII(B) receptor, binds the ligand and then transphosphorylates a second type RI receptor. The ligand-RII-RII complex is a functional serine threonine kinase, the functional targets of which are the members of the Smad cytoplasmic protein family (41).

Although specific activin receptor subunits have been identified and cloned, efforts to isolate an inhibin receptor have not been successful. Numerous studies were done to identify an inhibin receptor. The inability to identify an inhibin receptor using oligonucleotides directed against conserved regions of known activin receptors suggests that the inhibin receptor may differ from the activin receptor subunits. Numerous in situ ligand binding studies were done using a wide variety of tissues to localize inhibin binding sites that could be indicative of novel inhibin function and a source of potential inhibin receptor. In the ovary, inhibin-specific binding sites are associated with the granulosa cell (22). The ovary is capable of producing inhibins, activins, and follistatins in response to pituitary gonadotropins and to local growth regulatory factors (42). Inhibin A and inhibin B are released from the ovary and regulate pituitary FSH in a traditional endocrine feedback manner (43, 44). In addition to endocrine regulations, the follicular granulosa cell, theca, cell and oocyte are able to respond to inhibin and to activin, and these effects can be modulated by the binding protein follistatin. Activin stimulates granulosa cell proliferation, theca cell androgen production, and oocyte maturation (21, 32–35, 45, 46). Each of these effects may be coordinated through an independent inhibin receptor. In the testis, inhibin-specific binding sites are present on interstitial cells that are also positive for 3β-hydroxysteroid dehydrogenase, suggesting that these cells are the steroidally active Leydig cells (23).

In experiments described in this paper, we identified inhibin binding sites in the trigeminal ganglion and spinal ganglion of the developing rat embryo. The ability of inhibin to act on these sites requires that the ligand be present. Inhibin α-subunit mRNA is expressed in the somites of the embryonic rat on E12, in the dorsal root ganglion from E12 to E20 (47), and in the somites of the 10.5 day mouse embryo (48); immunoreactive α-subunit protein is localized in the somites of chick embryos (49). Moreover, both inhibin α- and β-subunit mRNAs are detected in different stages of the early mouse embryo and in embryonic stem and embryonic carcinoma cells that model early murine fetal development (50, 51). Likewise, human primary embryos have been shown to secrete immunoreactive α-subunit protein (52). Therefore, the developing embryo likely produces inhibin in restricted cellular sites, and this inhibin may have effects specifically on cellular loci such as the trigeminal ganglion and spinal ganglia, where inhibin-specific binding sites have been localized. Further analysis will be required to delineate the specific effect(s) of inhibin (and activin) on these cellular sites.

A powerful model system to delineate the physiological relevance of inhibin and activins is the genetic deletion of subunit genes through homologous recombination (the knockout mouse model). A series of animals deficient in the α- or β-subunits, in the receptors for activin, and in the activin/inhibin-binding protein, follistatin, have been generated (24, 53–57). Animals deficient in the inhibin α-subunit develop gonadal tumors (24–26), activin type RII receptor is down-regulated (57), and, as shown herein, the tumors bound iodinated inhibin A preferentially. Upon identification of the ovarian tumors as a tissue source of a potential inhibin receptor, we initiated studies to purify the protein using classical affinity chromatography methods. Four inhibin-binding proteins were partially purified. The receptor proteins were identified by cross-linking labeled ligand to putative receptor and examining the retardation of the complex on SDS-PAGE. Our inability to sequence the N terminus of the proteins isolated by affinity purification means that we can only speculate on the relationship between the proteins that bind iodinated inhibin. The two smaller proteins (complexes of 86 and 72 kDa) may be proteolytic cleavage products of the larger proteins. A broad spectrum mixture of protease inhibitors was used throughout the purification procedure; however, degradation of the larger proteins is a possibility. Alternatively, the larger molecular weight shifts may represent complexes of the smaller proteins with the iodinated inhibin. A third possibility is that several classes of inhibin receptor proteins exist in the tumor tissues. For example, inhibin may bind a yet-unidentified type II receptor, may bind and activate a type RI receptor, may have a structurally dissimilar receptor, or may use an adapter protein, such as type RII receptor, to act in concert (or competition) with activin type RII or RI receptors. The existence of an inhibin-specific receptor and competition with the activin receptor are not mutually exclusive conclusions. Indeed, an inhibin-specific protein band has been identified in the context of activin type RII and RI receptors in human erythroid precursor cells (16). Whether the protein identified in the K562 cell system is similar to one of the proteins identified in this study remains to be clarified. Indeed, the complete elucidation of the functional relationship between inhibin and activin receptors awaits the cloning of the inhibin receptor.

The fact that the inhibin receptor can be clearly identified in the knockout mouse tumor tissue is significant. It is known that one of the phenotypes of these tumors is low expression of type RII receptor, likely due to down-regulation by activin (24–25, 57). Similarly, the inhibin receptor may be up-regulated by persistent activin or by the lack of negative feedback of
Inhibin. Clearly, additional studies are necessary to determine what role, if any, activin and inhibin have in tumorigenesis and whether an inhibin receptor is expressed in human epithelial ovarian tumors.

In summary, inhibin binding moieties were found to be abundant in gonadal tumors that arise from the genetic elimination of the \( \alpha \)-subunit of inhibin. Four inhibin-binding proteins were purified by inhibin affinity chromatography from these tumors. Finally, the proteins eluted from the inhibin column were found to be distinct receptor proteins and not follistatin. Further work will be required to clone and characterize the inhibin receptor-generated proteins identified by affinity column purification; however, the results presented in this study represent an important first step toward the elucidation of the inhibin receptor and signal transduction system.

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