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Unique Recognition of Activin and Inhibin by Polyclonal Antibodies to Inhibin Subunits

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Inhibin-A is a glycoprotein composed of an α subunit containing a glycosylation site and a β_A subunit, whereas activin-A is a homodimer of two inhibin β_A subunits. We examined the recognition of activin-A and inhibin-A by several antisera to the α or β_A subunit, and factors affecting the recognition. A total of six polyclonal antibodies to inhibin subunits, *i.e.*, two antisera to a peptide fragment of the α subunit [α(1-19) and α(1-26)], and four antisera to the β_A subunit [β_A(1-10), β_A(70-79), β_A(87-99), and β_A(94-105)], was generated. On Western blot analysis, the anti-β_A(87-99) and β_A(94-105) sera recognized recombinant human activin-A but not inhibin-A under non-reducing conditions. When inhibin-A was deglycosylated with *N*-glycosidase-F, inhibin-A could be recognized by the anti-β_A(87-99) and β_A(94-105) sera. In addition, when activin-A bound to a nitrocellulose membrane was pre-incubated with recombinant human follistatin, the recognition of activin-A by the anti-β_A(87-99) and β_A(94-105) sera was decreased. These results suggested that the lower affinity of follistatin to inhibin-A than to activin-A might be likely explained as reflecting a site associated with the glycosylation of inhibin-A. However, the exposure of amino acids 87-105 of the inhibin β_A subunit on the molecular surface through deglycosylation did not increase the affinity of inhibin-A for follistatin but rather resulted in poor binding with follistatin. The present data suggest that (1) amino acids 87-105 of the inhibin/activin β_A subunit are located on the molecular surface, although this region of inhibin-A is concealed by the carbohydrate chain of the α subunit, (2) the region responsible for follistatin binding within the activin β_A subunit is spanned by amino acids 87-105, and (3) the mode of binding of inhibin-A to follistatin is quite different from that of activin-A to follistatin, and the former may be influenced by glycosylation.

Key words: activin, follistatin, inhibin, polyclonal antibodies, recognition.

Activins and inhibins, members of the transforming growth factor (TGF)-β superfamily, were purified from porcine and bovine follicular fluid as modulators of follicle-stimulating hormone (FSH) secretion from the pituitary gland (*1-6*). Inhibins are 32 kDa heterodimeric proteins composed of a common α subunit disulfide-bonded to either a β_A subunit, to form inhibin-A ($\alpha\beta_A$), or a β_B subunit, to form inhibin-B ($\alpha\beta_B$). Activins are 28 kDa homo- or heterodimeric proteins composed of either two β_A (or β_B) subunits of inhibin-A (or -B), to form activin-A (or -B), or the β_A- and β_B-subunits of inhibin-A and -B, to form activin-AB (see Refs. *7* and *8*). Recently, β_C- and β_D-subunits were cloned from human and *Xenopus* liver cDNAs (*9, 10*), and, therefore, it is likely that new types of activins or inhibins will be formed as homodimers or heterodimers with the α-, β_A-, and/or β_B-subunits.

Activins are believed to act as local regulators of cell differentiation and proliferation (*11*). Like other members of the TGF-β superfamily, activins exhibit potent activities in diverse biological processes, including promotion of erythroid differentiation (*12*), induction of mesoderm formation (*13*), stimulation of early embryogenesis (*14*), promotion of folliculogenesis (*15*), modulation of pituitary and pancreatic hormone release (*16-18*), and bone formation (*19*). In contrast, the action of inhibins' as local growth factors is less understood, although inhibin α mRNA was found to be widely distributed throughout the body (*20*), and inhibins have opposite effects to activins in some biological processes such as FSH secretion from the pituitary gland (*21-23*).

The results as to the localization and expression of activins or inhibins obtained on immunological analyses must be carefully interpreted because of the close structural relationship between these two types of proteins, *i.e.*, both activins and inhibins possess common β subunits, and not only the β subunit but also the α subunit belongs to the

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Abbreviations: ECL, enhanced chemiluminescence; FSH, follicle-stimulating hormone; 2ME, 2-mercaptoethanol; PVDF, polyvinylidene difluoride; rh, recombinant human; TBS-T, Tris-buffered saline containing 0.05% Tween-20; TGF, transforming growth factor.

TGF-β superfamily (*24*). In the present study, a total of six polyclonal antibodies to various synthetic peptides corresponding to mature regions of the α and β_A subunits of inhibins/activins formed by post-translational proteolysis was generated. At first, we examined the recognition of recombinant human- (rh-) activin-A and purified bovine inhibin-A by these antisera. Furthermore, to characterize the activin-A and inhibin-A molecules more clearly, the region responsible for the binding to follistatin, a monomeric protein known to bind to both activins and inhibins through the common β subunit (*25-27*), within activin-A or inhibin-A was probed using these antisera.

MATERIALS AND METHODS

Materials—Recombinant human-activin-A and rh-follistatin, purified from the culture supernatant of Chinese hamster ovary cells bearing an expression vector for the cDNAs of the peptides (*28, 29*), were kindly provided by Dr. Y. Eto (Ajinomoto, Kawasaki). Inhibin-A, purified from bovine follicular fluid by immunoaffinity chromatography (*30*), was kindly provided by Dr. Y. Hasegawa (Kitasato University, Towada). Recombinant human-TGF-β_1 and -β_2 were purchased from King Brewing (Kakogawa) and Genzyme (Cambridge, MA), respectively.

Production of Antisera—To generate two antisera directed against the α subunit, and four antisera directed against the β_A subunit, synthetic peptides comprising residues 1-19, Tyr^{20} [α(1-19)], and 1-26 [α(1-26)] of the porcine α subunit, and residues 1-10, Tyr^{11} [β_A(1-10)], 70-79 [β_A-(70-79)], 87-99 [β_A(87-99)], and 94-105 [β_A(94-105)] of the human β_A subunit were purchased from Multiple Peptide Systems (San Diego, CA) (Table I). The peptides were partially purified by gel filtration chromatography on a Sephadex G-25 column equilibrated with 0.5% acetic acid in water. Better than 98% purity was obtained by reverse-phase HPLC with a gradient of increasing concentration of acetonitrile in water containing 0.03% trifluoroacetic acid. The purified product was determined to have the correct amino acid composition. The β subunit is evolutionally conserved, and the amino acid sequence of the mature region of the β_A subunit formed through post-translational proteolysis is identical in man, pig, cattle, and rat (see Ref. *7*). In addition, the amino acid sequence of the β_B subunit is identical in man and cattle, and only one residue among 115 amino acids was found to be different between cattle and pig (*31*). Peptides α(1-19) and β_A(1-10) were coupled to BSA with bisdiazotized benzidine, and β_A(87-99) was coupled to BSA with carbodiimide (*32*). The α(1-26), β_A(70-79), and β_A(94-105) peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde.

New Zealand white rabbits (generally two rabbits per peptide) were injected at a total of 20-30 intradermal sites with 1-2 mg of a conjugated peptide as an emulsion in 50% Freund's complete adjuvant, in a total volume of 2 ml. The rabbits were boosted every 3-4 weeks with 0.5-1 mg of the conjugated peptide in 50% Freund's incomplete adjuvant. Blood was taken 10 days after the last boosters. The reactivity of antisera was monitored by ELISA. A total IgG fraction was prepared from each antiserum by elution through a protein A-Sepharose column (Pharmacia-LKB, Sweden) (*33*).

Western Blot Analysis—One hundred nanogram rh-activin-A, purified bovine inhibin-A, rh-TGF-β_1, or rh-TGF-β_2 dissolved in SDS-PAGE sample buffer was loaded on a 12.5% polyacrylamide gel as described by Laemmli (*34*). Proteins were heated with or without 4% (w/v) 2-mercaptoethanol (2ME) for 5 min at 94°C. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) in a semi-dry blotting apparatus (Semi-dry transfer cell; Bio-Rad, Richmond, CA). The membranes were blocked with skim milk (Block Ace; Snow Brand, Tokyo) for 1 h, and then incubated with the anti-α or β_A serum diluted 50 to 3,000-fold with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 16 h at 4°C. The membranes were then washed with TBS-T (1 h), and reacted with a peroxidase-conjugated anti-rabbit IgG antibody (EY Laboratories, San Mateo, CA) diluted 1,000-fold with TBS-T for 40 min at room temperature. After washing with TBS-T (1 h), the proteins that reacted were visualized with a commercial kit (ECL detection kit, Amersham, UK) using an enhanced chemiluminescence (ECL) reagent.

Changes in Recognition on Pre-Incubation with Follistatin—The effect of the activin-follistatin complex on the recognition of activin-A by the antisera was first examined by immunoreaction after incubation with rh-follistatin on Western blots (trial 1). Three hundred ng rh-activin-A was subjected to SDS-PAGE and then transferred to a PVDF membrane. After blocking to prevent non-specific binding, the membrane was incubated with TBS-T containing 50 μg/ml rh-follistatin and 250 μg/ml rat albumin for 16 h at 4°C, and then incubated with the antisera for a further 24 h at 4°C after removal of the rh-follistatin and rat albumin. The detection of bands was carried out using the peroxidase-conjugated anti-rabbit IgG antibody and the ECL reagent. As a control, the membrane was incubated with TBS-T containing only rat albumin during the first incubation.

Next, to prevent possible protein loss during SDS-PAGE

TABLE I. **Amino acid sequences of synthetic peptide fragments of α and β_A subunits of activin/inhibin used to generate antisera and their homology to related peptides.** The homology was calculated with reference to Vale *et al.* (*7*), Hötten *et al.* (*9*), Oda *et al.* (*10*), and Thompson *et al.* (*31*). The symbol, —, denotes "not calculable."

Name of peptide	Amino acid sequence	Species	% homology to:						
			Porcine α	Bovine α	Human β_A	Bovine β_A	Bovine β_B	Human β_C	*Xenopus* β_D
α(1-19)	STAPLPWPWSPAALRLLQR(Y)	Porcine	100	95	—	—	—	—	—
α(1-26)	STAPLPWPWSPAALRLLQRPPEEPAV	Porcine	100	88	—	—	—	—	—
β_A(1-10)	GLECDGKVNI(Y)	Human	—	—	100	100	70	30	40
β_A(70-79)	GHSPFANLKS	Human	—	—	100	100	30	10	10
β_A(87-99)	RPMSMLYYDDGQN	Human	—	—	100	100	62	62	62
β_A(94-105)	YDDGQNIIKKDI	Human	—	—	100	100	50	58	42

and transfer to the membrane, the effect of pre-incubation with follistatin on the recognition of activin-A by the antisera was evaluated by slot blot analysis (trial 2). Because the results of trial 1 indicated that only non-reduced rh-activin-A could bind to rh-follistatin, and because the anti-β_A(1-10) and β_A(70-79) sera did not react with the activin-follistatin complex, the anti-β_A(87-99) and β_A(94-105) sera were used in this assay. Five nanogram rh-activin-A was directly bound to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and then incubated with TBS-T containing 166 ng/ml rh-follistatin and 833 ng/ml rat albumin for 3 h at room temperature after blocking. As a control, the membrane was incubated with TBS-T containing 833 ng/ml rat albumin after blocking. Then, the membrane was reacted with the antiserum for 1 h. The proteins that reacted were visualized using the peroxidase-conjugated anti-rabbit IgG antibody and the ECL reagent.

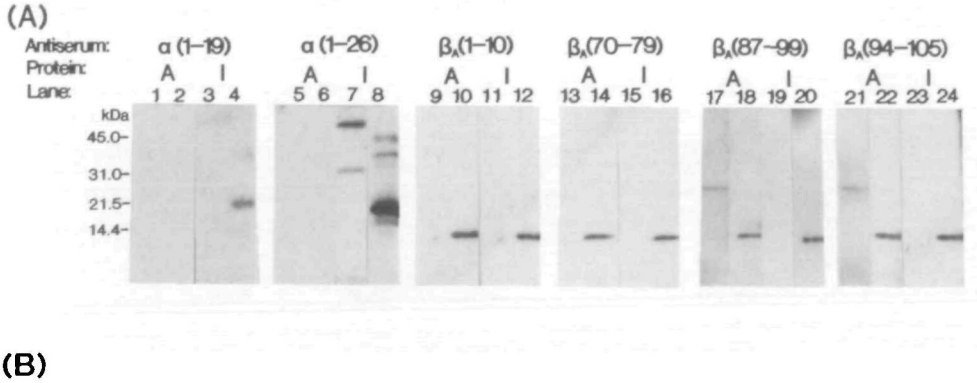
Bands were loaded into a personal computer (Apple, Tokyo) using a scanner (Color image scanner GT-4000; Epson, Tokyo), and the intensity of the bands was quantified with NIH-image software. The binding assays were repeated at least three times, and the results were expressed as a ratio to the intensity of the control. The statistical difference between treatments was evaluated by means of Student's *t* test.

Deglycosylation of the Inhibin α Subunit—The bovine inhibin α subunit contains one potential asparagine-linked glycosylation site within its mature region (see Ref. 7). To examine the effect of deglycosylation of inhibin on the recognition of inhibin-A by the antisera and the affinity of inhibin-A to rh-follistatin, purified bovine inhibin-A was digested with *N*-glycosidase-F (*35*) from *Flavobacterium meningosepticum* (Boehringer Mannheim, Indianapolis, IN). Two hundred nanogram purified bovine inhibin-A was incubated with 1 U enzyme in 2 μl of 10 mM phosphate-buffered saline and 2.5 mM EDTA (pH 7.4) for 24 h at 37°C. To eliminate the possibility of non-specific degradation of the protein during the incubation, purified bovine inhibin-A or rh-activin-A was incubated with the solution without the endoglycosidase enzyme for 24 h at 37°C. The digested products were subjected to Western blot analysis and follistatin binding analysis.

The affinity to rh-follistatin was evaluated by a technique similar to slot blot analysis, as described above. Thirty nanogram purified bovine inhibin-A, deglycosylated inhibin-A, or rh-activin-A was bound to a nitrocellulose membrane, and then incubated with TBS-T containing 125 ng/ml rh-follistatin, 625 ng/ml rat albumin, and antiserum directed against a synthetic human follistatin peptide (amino acids 123-134 or 300-315) (*36*) for 16 h at 4°C after blocking with skim milk. The binding of rh-follistatin was visualized using the peroxidase-conjugated anti-rabbit IgG antibody and the ECL reagent. Relative band intensity was calculated as the ratio of the band intensity to that of purified bovine inhibin-A. Statistical analyses were performed as described above.

RESULTS

Recognition of Activin-A and Inhibin by Antisera—The results of recognition of activin-A and inhibin-A by the antisera on Western blots are shown in Fig. 1. No bands of activin-A and inhibin-A were detected, when normal rabbit serum instead of anti-α or β_A serum was used (data not shown). The anti-β_A(1-10) and β_A(70-79) sera reacted with reduced activin-A (14 kDa), while no bands were detected with activin-A up to 500 ng under non-reducing conditions. In contrast, the anti-β_A(87-99) and β_A(94-105) sera readily recognized both non-reduced (25 kDa) and reduced activin-A, although the reactivity to reduced activin-A was stronger. None of the antisera directed against the β_A subunit reacted with non-reduced inhibin-A. The antisera did not



(B)

Summary of recognition of rh-activin-A and purified bovine inhibin-A by antisera

Antiserum:	α(1-19)	α(1-26)	β_A(1-10)	β_A(70-79)	β_A(87-99)	β_A(94-105)
Activin-A						
2ME −	—	—	—	—	+	+
+	—	—	+	+	+	+
Inhibin-A						
2ME −	—	+	—	—	—	—
+	+	+	+	+	+	+

Fig. 1. **Recognition of rh-activin-A and purified bovine inhibin-A by the antisera.** (A) The immunoreaction of rh-activin-A and purified bovine inhibin-A on Western blotting. One hundred nanogram rh-activin-A or purified bovine inhibin-A was subjected to SDS-PAGE and then transferred to a membrane The membrane was incubated with antisera directed against synthetic peptides of various fragments of inhibin subunits after blocking to prevent non-specific binding. The proteins that reacted were visualized using a second antibody and the ECL reagent. A and I indicate rh-activin-A and purified bovine inhibin-A, respectively. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23 correspond to non-reducing conditions (2ME−) on SDS-PAGE, and lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 correspond to reducing conditions (2ME+). (B) Summary of the antibody recognition results.

clearly cross-react with TGF-β_1 or -β_2. When anti-β_A(1-10) serum was incubated with reduced TGF-β_1, a band could be detected at 12.5 kDa, although its intensity was quite weak (data not shown).

The anti-α(1-26) serum recognized both non-reduced (34 kDa) and reduced (20 kDa) inhibin-A, but the anti-α(1-19) serum did not recognize inhibin-A up to 500 ng under non-reducing conditions. In addition, anti-α(1-26) serum detected a higher molecular weight form of inhibin-A (*37*) of 52 kDa. Both the anti-α(1-19) and α(1-26) sera reacted with neither activin-A nor TGF-β_1 or -β_2. These results might be due to a difference in the recognition region of the inhibin α subunit between the anti-α(1-19) and anti-α(1-26) sera. Bisdiazotized benzidine, a cross-linker between carboxyl-terminal sequences, was used to link the peptide to a carrier protein when developing the anti-α(1-19) serum. On the other hand, glutaraldehyde, a cross-linker between amino-terminal sequences, was used to generate the anti-α(1-26) serum. Therefore, the region near the amino-terminal sequence of the α subunit might be recognized by the anti-α(1-19) serum, but not by the anti-α(1-26) serum. This possibility then suggests that the region located on the molecular surface is not in the amino-terminal sequence, but in a sequence further into the protein, perhaps including amino acids 20-26.

Effect of Pre-Incubation with Follistatin on Recognition of Activin-A by the Antisera—To explore the binding sites of the activin β_A subunit for follistatin, activin-A bound to a membrane was first incubated with follistatin and then reacted with the antisera. If the region of the β_A subunit responsible for follistatin binding coincides with the epitope of the antisera, the recognition of activin-A by the antisera should be decreased. In trial 1, the effect of follistatin binding was examined using Western blots. First, follistatin binding to activin-A was detected with the antisera directed against a synthetic human follistatin peptide (amino acids 123-134 or 300-315) (*36*). Activin-A was electrophoresed under non-reducing conditions, and then reacted with the anti-β_A(87-99) and β_A(94-105) sera, since follistatin does not bind to reduced activin-A, and since both the anti-β_A(1-10) and β_A(70-79) sera cannot detect the activin-follistatin complex (data not shown). In addition, a preliminary study indicated that these antisera reacted with neither follistatin nor rat albumin (data not shown).

The changes in the intensity of the band corresponding to activin-A on pre-incubation with follistatin are shown in Fig. 2. When activin-A was subjected to SDS-PAGE and then transferred to a membrane (trial 1), the band intensity of activin-A on detection with the anti-β_A(94-105) serum showed a 30% decrease on pre-incubation with follistatin. The incubation with follistatin had less effect on the recognition by the anti-β_A(87-99) serum (Fig. 2A). In contrast, when the protein was directly bound to the membrane (trial 2), the recognition of activin-A by the anti-β_A(87-99) and β_A(94-105) sera was slightly but significantly decreased on the addition of follistatin (Fig. 2B). The variability among assays in trial 2 was much smaller than that in trial 1. The decrease in band intensity on slot blots did not result from non-specific inhibition of immunoreac-

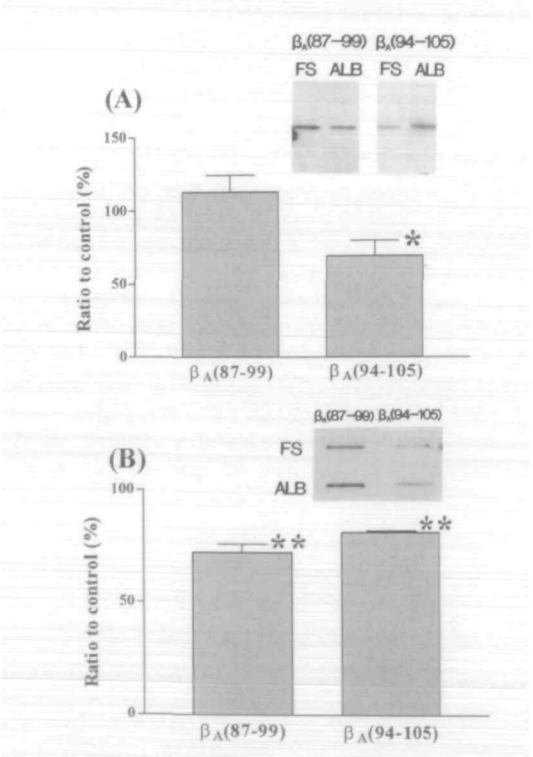


Fig. 2. **Changes in recognition of rh-activin-A after incubation with rh-follistatin and the antisera.** (A) Results of trial 1. Three hundred nanogram rh-activin-A was electrophoresed and then transferred to a PVDF membrane. The membrane was incubated with 50 μg/ml rh-follistatin and 250 μg/ml rat albumin after blocking to prevent non-specific binding. As a control, incubation was carried out with only 250 μg/ml rat albumin after blocking. Then, the anti-β_A (87-99) or β_A(94-105) serum was incubated, and the proteins that reacted with the antiserum were detected using a second antibody and the ECL reagent. (B) Results of trial 2. Five nanogram rh-activin-A was directly bound to a nitrocellulose membrane, and then reacted with 166 ng/ml rh-follistatin and 833 ng/ml rat albumin after blocking. As a control, incubation was carried out with only 833 ng/ml rat albumin after blocking. The membrane was then reacted with the antiserum, and the proteins that reacted were visualized using a second antibody and the ECL reagent. Relative band intensity was calculated as the ratio of the band intensity of rh-activin-A incubated with rh-follistatin to that of the control in the same assay. The symbols, * and **, indicate significant differences from the control data at $p<0.05$ and $p<0.01$, respectively. The abbreviations, FS and ALB, denote "rh-follistatin" and "rat albumin," respectively.

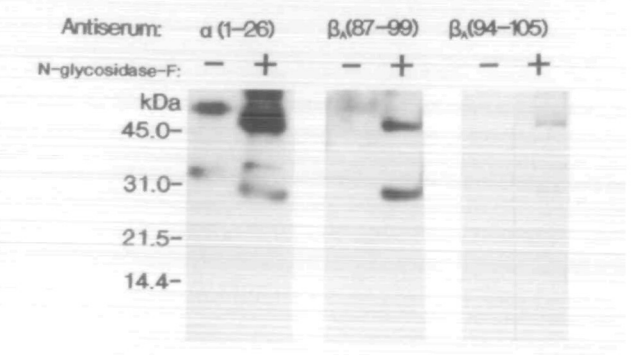


Fig 3. **Recognition by the antisera of purified bovine inhibin-A after deglycosylation.** For cleavage of the carbohydrate chain of the inhibin a subunit, 200 ng purified bovine inhibin-A was incubated with 1 U *N*-glycosidase-F for 24 h at 37°C. The digested products were subjected to Western blot analysis using the anti-α(1-26), β(87-99), or β(94-105) serum. The proteins that reacted were visualized using a second antibody and the ECL reagent.

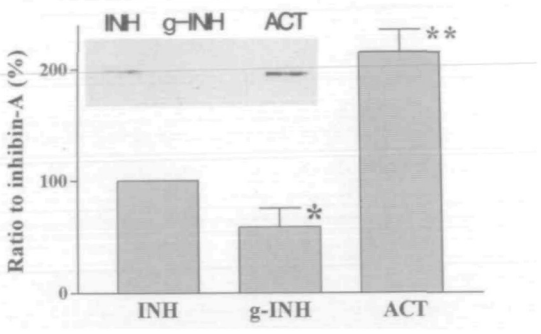


Fig. 4. **Affinity of purified bovine inhibin-A, deglycosylated inhibin-A, and rh-activin-A to rh-follistatin.** To examine the effect of cleavage of the carbohydrate chain of the inhibin α subunit on follistatin binding, 30 ng purified bovine inhibin-A was incubated with 150 mU *N*-glycosidase-F for 24 h at 37°C. Thirty nanogram purified bovine inhibin-A, deglycosylated inhibin-A, or rh-activin-A was directly bound to a nitrocellulose membrane, and then reacted with 125 ng/ml rh-follistatin, 625 ng/ml rat albumin, and antiserum directed against a synthetic human follistatin peptide (amino acids 300-315) (*36*) for 16 h at 4°C after blocking to prevent non-specific binding. The proteins that reacted were visualized using a second antibody and the ECL reagent. Relative band intensity was calculated as the ratio of the band intensity to that of purified bovine inhibin-A. The symbols, * and **, indicate significant differences from the purified bovine inhibin-A data at $p<0.05$ and $p<0.01$, respectively. The abbreviations, INH, g-INH, and ACT, denote "purified bovine inhibin-A," "deglycosylated inhibin-A," and "rh-activin-A," respectively.

tivity during the incubation with follistatin, because the recognition of activin-A by another series of anti-activin-A antisera raised in chicken (*38*) was not affected by incubation with follistatin (IgY #2: 99.0±4.4, $p>0.05$; IgY #3: 114.2±8.4, $p>0.05$). The immunoreactivity of activin-A after incubation with follistatin with the anti-β_A(87-99) serum on slot blots was not consistent with that found on Western blots, whereas similar results were obtained with both systems using the anti-β_A(94-105) serum. The reason for this discrepancy is not known.

Effect of Deglycosylation of Inhibin-A on Recognition by the Antisera and Follistatin Binding—To examine the effect of the carbohydrate chain of the inhibin α subunit on the recognition by the antisera, deglycosylation of inhibin-A was carried out with *N*-glycosidase-F. The recognition of deglycosylated inhibin-A by the antisera is shown in Fig. 3. None of the antisera reacted with *N*-glycosidase-F (data not shown). When inhibin-A was deglycosylated, the anti-β_A(1-10) and β_A(70-79) sera did not react with the deglycosylated inhibin-A, and the anti-α(1-19) serum weakly detected higher molecular weight forms of inhibin-A (48 and 92 kDa) under non-reducing conditions (data not shown). The anti-α(1-26) serum could detect inhibin-A under non-reducing conditions, as shown in Fig. 1, and also detected deglycosylated inhibin-A (Fig. 3). Western blot analysis of deglycosylated inhibin-A showed slightly faster migration than for non-reduced inhibin-A (30 and 48 kDa *vs.* 34 and 52 kDa), indicating effective deglycosylation, as previously reported (*39, 40*). Deglycosylation of inhibin-A enabled the anti-β_A(87-99) and β_A(94-105) sera to detect bands at 30 and 48 kDa (Fig. 3), although these antisera never reacted with inhibin-A under non-reducing conditions (Fig. 1), suggesting that, at least, amino acids 87-105 of the inhibin β_A subunit may be obscured by the asparagine-linked carbohydrate chain of the inhibin α subunit. Considering that this region within the activin β_A subunit is responsible for follistatin binding, the observation that follistatin binding to inhibin-A is much lower than that to activin-A (*25, 41*) may be explained by the masking of amino acids 87-105 of the inhibin β_A subunit by the carbohydrate chain.

To examine this possibility, the effect of deglycosylation of inhibin-A on its affinity to follistatin was examined by slot blot analysis. In a preliminary study, when biotinylated follistatin was incubated with inhibin-A or activin-A, significant reaction with neither inhibin-A nor activin-A was detected. Therefore, inhibin-A or activin-A bound to nitrocellulose was incubated with rh-follistatin, and the binding was detected with the antisera directed against the synthetic peptide of human follistatin fragment 123-134 or 300-315 (*36*). As shown in Fig. 4, the affinity of inhibin-A to follistatin was significantly lower than that of activin-A, which was consistent with the results obtained on Western-ligand blot analysis by Miyanaga *et al.* (*41*). Unexpectedly, the cleavage of the carbohydrate chain of the inhibin α subunit resulted in lowered affinity to follistatin, indicating that the carbohydrate chain of the inhibin α subunit may participate in follistatin binding to inhibin.

DISCUSSION

The recognition of activin-A by antisera directed against four different regions of the inhibin/activin β_A subunit was examined by Western blot analysis. The anti-β_A(1-10) and β_A(70-79) sera could not detect non-reduced activin-A up to 500 ng, although the anti-β_A(87-99) and β_A(94-105) sera did react with non-reduced activin-A. However, all of them reacted with reduced activin-A, suggesting that the anti-β_A(1-10) and β_A(70-79) sera could only bind as to the β_A subunit on the cleavage of disulfide bonds of activin-A. Amino acids 1-10 and 70-79 of the β_A subunit might, therefore, not be located on the surface of the activin-A molecule, due to the presence of intra- and intermolecular disulfide bonds, respectively. In contrast, at least in part, amino acids 87-105 may be located on the molecular surface, allowing recognition by the antisera. Analysis of the crystal structure of TGF-β_2 (*42, 43*), which shares 37% homology with the human β_A subunit of inhibin/activin (*24*), allows us to deduce that amino acids 1-10 of the activin β_A subunit make one α helical structure and include one intramolecular disulfide bond. Amino acids 70-79 of the activin β_A subunit would be located near a cysteine residue participating in an intermolecular bond. The cysteine residue located at amino acid position 80 appeared to be involved in the intermolecular bond, because site-directed mutation at cysteine^{80} of the β_A subunit prevented dimer formation (*44*). In addition, the monomer of the β_A subunit of inhibin/activin isolated from bovine follicular fluid has a free cysteine residue at amino acid position 80, indicating that this cysteine residue would be involved in intermolecular bonding (*45*). Other site-mutation studies suggested that cysteines 4 and 12, and cysteines 11 and 81 of the activin β_A subunit formed intramolecular disulfide bonds (*44*).

Unexpectedly, all the antisera to the β_A subunit including the anti-β_A(87-99) and β_A(94-105) sera could not detect

inhibin-A under non-reducing conditions. The fact that the anti-β_A(87-99) and β_A(94-105) sera could recognize activin-A but not inhibin-A under non-reducing conditions suggests differences in the three-dimensional structure of the β_A subunit or of regions located on the molecular surface of the β_A subunit between inhibin-A and activin-A. The differences in recognition by the antisera between inhibin-A and activin-A could be partly explained by glycosylation of the molecules, because both the anti-β_A(87-99) and β_A(94-105) sera could detect non-reduced inhibin-A after cleavage of the carbohydrate chain of the inhibin α subunit. This observation suggests that the carbohydrate chain of the α subunit may conceal the region recognized by the anti-β_A(87-99) and β_A(94-105) sera, *i.e.*, amino acids 87-105.

Accumulating evidence indicates that the multiple biological effects of activin can be neutralized by follistatin at the local level (*25, 46, 47*). In fact, follistatin preferably binds to activin-A with high affinity (*25, 27, 41*). Therefore, the effect of follistatin binding to activin-A on the recognition of activin-A by the antisera was examined. Our data suggest that, at least, amino acids 87-105 of the activin β_A subunit are involved in follistatin binding to activin-A, because the recognition of activin-A by the anti-β_A(87-99) and β_A(94-105) sera was decreased by pre-incubation with follistatin. These results are partly consistent with the results of Schneyer *et al.* (*27*), which indicated that the sites of the activin β_A subunit involved in follistatin binding are amino acids 15-29 near the N-terminus and, within the C-terminal, 99-116. They based their conclusion on changes in activin binding to follistatin after incubation with synthetic fragments of the β_A subunit. Deducing the three-dimensional conformation of activin-A, they suggested that follistatin could recognize one epitope from each subunit of the activin-A molecule, because the sequence of amino acids 99-116 of one β_A subunit would be juxtaposed to the N-terminus of the disulfide-linked homologous subunit, including amino acids 15-29 (*27*).

Follistatin can bind to inhibin-A through the common β_A subunit (*26, 27*), with much lower affinity than to activin-A (*25, 27*). The lower affinity to inhibin-A might be due to either the difference in the number of binding sites between inhibin-A and activin-A (1 *vs.* 2) (*41*) or the requirement for a dimeric form of the β_A subunit for complete binding (*27*), or both. If such constraints are involved in the poor affinity of inhibin-A to follistatin, it can be hypothesized that exposure of amino acids 87-105 of the inhibin β_A subunit on the molecular surface through deglycosylation would cause an increase or no change in the affinity of inhibin-A to follistatin. Amino acids 87-105 of the β_A subunit of inhibin/activin form a unique region, *i.e.*, in the activin β_A subunit, this region being responsible for the binding with follistatin, whereas in the inhibin β_A subunit, it is concealed by the carbohydrate chain of the α subunit. However, cleavage of the carbohydrate chain of the inhibin α subunit resulted in a reduction of the affinity to follistatin, suggesting that the mode of binding of inhibin-A with follistatin is quite different from that of activin-A with follistatin. The carbohydrate chain of inhibin-A itself might be important for binding with follistatin.

Does the reduced affinity of deglycosylated inhibin-A for follistatin have any physiological relevance? Generally, glycosylation affects the stability of a molecule, resulting in an increase in its biological half-life. Cleavage of the carbohydrate chain might increase clearance of the protein, as has been suggested for gonadotropin (*48, 49*). Alternatively, one could infer that inhibin activity might be regulated by inhibin α subunit endoglycosidase activity in some biological systems. Some fraction of inhibins exists in the form of an inhibin-follistatin complex in serum and follicular fluid (*27, 50, 51*). Once an endoglycosidase cleaves the carbohydrate chain of the inhibin α subunit, inhibins would be unable to bind with follistatin because of a change in their conformation. Free inhibins liberated from follistatin might easily bind with putative inhibin-specific receptors, resulting in the transduction of their signals. There is evidence that the binding of FSH to FSH-responsive tissues is increased by deglycosylation (*52*). In addition, recent evidence indicates that the asparagine-linked carbohydrate chain of the α subunit of human chorionic gonadotrophin prevents the molecule from combining with free β subunits in the extraembryonic coelomic fluid (*53*).

In summary, in the present study six polyclonal antibodies to mature regions of the α and β_A subunits of inhibin/activin were generated and characterized by Western blot analysis. We demonstrated that (1) the recognition of rh-activin-A or purified bovine inhibin-A by the antisera is unique and dependent on the peptide region used as the antigen, suggesting that these antisera are useful not only for examining the localization but also for quantitative analyses of the activin and inhibin proteins, separately, (2) amino acids 87-105 of the activin β_A subunit are at least one of the crucial sites for binding with follistatin, whereas this region in inhibin-A is concealed by the carbohydrate chain of the inhibin α subunit, and (3) however, the exposure of amino acids 87-105 of the inhibin β_A subunit to the molecular surface on deglycosylation results in reduced affinity of inhibin-A for follistatin. Considering that the affinity of inhibin-A to follistatin varied in response to the presence of the carbohydrate chain, glycosylation would be likely to play an important role in the inhibin action and/or in activin-inhibin-follistatin systems.

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