Production and purification of recombinant human inhibin and activin

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Abstract

Inhibin and activin are protein hormones with diverse physiological roles including the regulation of pituitary FSH secretion. Like other members of the transforming growth factor-β gene family, they undergo processing from larger precursor molecules as well as assembly into functional dimers. Isolation of inhibin and activin from natural sources can only produce limited quantities of bioactive protein. To purify large-scale quantities of recombinant human inhibin and activin, we have utilized stably transfected cell lines in self-contained bioreactors to produce protein. These cells produce approximately 200 μg/ml per day total recombinant human inhibin.

Conditioned cell media can be purified through column chromatography resulting in dimeric mature 32–34 kDa inhibin A and 28 kDa activin A. The purified recombinant proteins maintain their biological activity as measured by traditional in vitro assays including the regulation of FSH in rat anterior pituitary cultures and the regulation of promoter activity of the activin-responsive promoter p3TP-luc in tissue culture cells. These proteins will be valuable for future analysis of inhibin and activin function and have been distributed to the US National Hormone and Peptide Program.

Journal of Endocrinology (2002) 172, 199–210

Introduction

Inhibin is a gonadal peptide originally isolated from ovarian follicular fluid (Ling et al. 1985, Miyamoto et al. 1985, Robertson et al. 1985). Sertoli cells in the male and granulosa cells in the female are the predominant production sites for the dimeric inhibin protein (Meunier et al. 1988). However, the inhibin subunits have been localized to other tissues (Roberts et al. 1989, Petraglia et al. 1990, Leung et al. 1998, Billiar et al. 1999). The related protein activin has a wider expression profile including the hypothalamus, pituitary gland, adrenal gland and placenta (Meunier et al. 1988, Petraglia et al. 1990, Roberts et al. 1992, 1996, Spencer et al. 1992, Wilson & Handa 1998).

Inhibin and activin are functional antagonists: inhibin suppresses while activin activates the release of follicle-stimulating hormone (FSH) from the anterior pituitary gland (Rivier et al. 1986, Rivier & Vale 1991, Woodruff et al. 1993).

Like all members of the transforming growth factor-β (TGF-β) superfamily, the inhibins and activins are processed from larger precursor proteins (Gray & Mason 1990, DuBois 1994, Robertson et al. 1997). Multiple proteases control different family member processing. TGF-β is processed by the subtilisin-like proprotein convertase, furin, which processes the pro-protein at multibasic residues (Dubois et al. 2001, Leitlein et al. 2001). The subtilisin-like proprotein convertases also cleave other TGF-β family members such as Mullerian-inhibiting substance and Lefty but the proteases responsible for the inhibin and activin processing are unknown (Nachtigal & Ingraham 1996, Ulloa et al. 2001). Inhibin is a dimer of one of several β-subunits designated A–E and a unique α-subunit (Pangas & Woodruff 2000). The predominant isoforms, inhibin A and inhibin B, are designated by the β-subunit. Activin is the homo- or heterodimer of the β-subunit and the nomenclature again reflects the β-subunit. Both proteins have maintained a high degree of conservation throughout mammalian evolution (Burt & Law 1994, Newfeld et al. 1999). Thus, human recombinant activin and inhibin can substitute for most mammalian orthologs as the amino acid conservation of the mature protein is 100% for the β-subunit in all known cloned mammalian species and 82–88% for the α-subunit.

For in vitro and some in vivo analyses of inhibin and activin function, purified recombinant proteins are required. The processing and secretion necessary for generating biologically active proteins poses multiple problems for recombinant inhibin and activin production. First, a complex pattern is generated from the processing and assembly of prepro- and mature hormones (Fig. 1). The human precursor α-subunit is 348 amino acids...
composed of a 43 amino acid pro-region, 171 amino acid αN region, and a 134 amino acid αC ‘mature’ region (Mason et al. 1996). The β-subunit precursor consists of a 290 amino acid pro-region and 116 amino acid C-terminus (Mason et al. 1996). While the partially processed α-subunit (‘αN-αC’ or ‘pro-αN-αC’) when found as a dimer with a mature βA subunit is biologically active, the β-subunit dimers must be fully processed to be functional (Mason et al. 1996). Secondly, the inhibins are post-translationally modified by glycosylation (Tierney et al. 1990). Mutagenesis of the glycosylation sites on inhibin α-subunit results in a secreted protein approximately 27 kDa in size (Mason et al. 1996). While it is unknown whether non-glycosylated inhibin maintains biological activity, blocking the multiple glycosylation sites on TGF-β results in non-secreted and inactive protein products (Brunner et al. 1992). Thirdly, activin is produced as a bioproduct of inhibin production. Since the biological functions are antagonistic, it is important that purified preparations be completely free of contamination from the other protein.

Strategies for purification must take into account the need for dimerization, cleavage and glycosylation to produce fully functional proteins. Bacterial overexpression is a common means to produce large quantities of recombinant proteins; however, no bacterial system can meet these processing requirements. Affinity purification that exploits epitope-tagged proteins also is a well-characterized means to generate large quantities of protein. However, for inhibin and activin, N-terminal tags are removed during normal processing. In addition, C-terminal epitope tags on the α-subunit render the protein unprocessable, similar to the inability of a C-terminally modified TGF-β to dimerize and be proteolytically processed (Wakefield et al. 1996).

Figure 1 Diagrammatic representation of inhibin (left) and activin (right) subunit assembly. (A) Structure of inhibin α- and inhibin/activin β-subunits. (B) Dimerization and cleavage of the two subunits give multiple protein products. Activin is a dimer of the β-subunits and inhibin is a dimer of the α- and β-subunits. Sizes are indicated to right. Not all the glycosylated forms are represented. (C) Mature inhibin A and mature activin A are dimeric C-terminal cleavage products of 32–34 kDa and 28 kDa respectively.

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Journal of Endocrinology (2002) 172, 199–210

Described here is the production and purification of inhibin A and activin A. To produce large quantities of protein, we have used stably transfected cell lines that produce human inhibin A and activin A. A biochemical approach coupled with affinity chromatography was used to purify inhibin A. Affinity chromatography was used to purify activin A. Both methods have produced milligram quantities of proteins useful for studying their biological functions. These methods could be extended to purification of the other inhibin and activin isoforms: inhibin B, activin B, and possibly activin AB. Inhibin A and activin A have been distributed to the US National Hormone and Peptide Program.

Materials and Methods

Cell culture

Cell lines that produce recombinant human inhibin A and activin A were obtained from Genentech Inc. (South San Francisco, CA, USA) and modified for adherent and bioreactor cultures (inhibin A cell line, NU-INHA1-BR; activin A cell line, NU-ACTA1-TF). These cells are Chinese hamster ovary (CHO) cells with integrated bi-cistronic expression plasmids for the inhibin α- and activin β-subunits. Cells were maintained in DME-F12 (1:1) medium (Gibco-Life Technologies, Rockville, MD, USA), 5% heat-inactivated fetal bovine serum, 1% penicillin–streptomycin and methotrexate. Cells were grown to confluence in growth media and passaged into flasks (Nunclon Delta flasks; Nunc, Rochester, NY, USA) at a density of \(7 \times 10^6\) cells/flask or into Cell Pharm-100 bioreactors (Unisyn Technologies, South Hampton, MA, USA) at a seeding density of \(6 \times 10^7\)/bioreactor. In the bioreactors, cell density was approximately constant but could reach 1-5 times the original density. In the flasks, cell number reached approximately 3 times the seeding density. During protein production, cells were grown serum-free. Medium was collected three to seven times a week depending on the cell line and production system. Cells were maintained for production for no longer than 1 month. Once collected, the medium was stored at \(-80^\circ\)C until purified. Upon thawing, the medium was concentrated to small volumes (i.e. less than 100 ml) and diafiltered on a tangential flow concentrator (Miniplate Concentrator; Millipore Corporation, Bedford, MA, USA) for use in chromatography. Protein production was monitored by weekly non-reducing protein blot analysis for the secreted proteins and ELISAs for total ligand production.

Ion-exchange chromatography

All purification steps were carried out using an FPLC system (Amersham-Pharmacia, Piscataway, NJ, USA). Chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless indicated. S-Sepharose FF resin (Amersham-Pharmacia), a strong cation-exchange column, was utilized as the first purification step. The sample was equilibrated in the column buffer (25 mM 2-morpholinoethanesulfonic acid (MES), 1 mM EDTA, pH 6.0), loaded onto the column, washed in column buffer, and eluted in a series of two elution buffers (buffer 1, 35 mM ‘GEB’ (35 mM MES, 35 mM 3-morpholinoopanesulfonic acid, 35 mM glycine, 35 mM Tris-HCl, 35 mM triethanolamine–HCl, 35 mM NaCl, pH 6.0); buffer 2, 1.5 M urea, 20 mM ‘GEB’, pH 7.5). Ten-milliliter fractions were collected and fractions containing the recombinant proteins were identified by immunoblotting. Those containing inhibin A were pooled and concentrated by tangential flow (Miniplate Concentrator).

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was performed through a phenyl-Sepharose CL-4B resin (Amersham-Pharmacia). The column was equilibrated in 0.5 M sodium citrate, 0.05 M Tris, pH 7.5. The conductivity of the sample was adjusted to 35 mΩ with 1-4 M sodium citrate, 0.1 M Tris, pH 7.5. After loading the sample, the column was washed in equilibration buffer and eluted in a linear gradient to a final eluent concentration of 4 M urea, 0.05 M Tris-HCl, pH 7.5. Fractions containing the eluted proteins were analyzed by immunoblotting. These fractions were pooled and concentrated first by tangential flow (Miniplate Concentrators) then by centrifugal filtration (Ultrafree-15 Centrifugal Filter, nominal molecular mass limit 10 kDa; Millipore) to less than 4 ml for size-exclusion chromatography.

Size-exclusion chromatography

A 2.5 × 90 cm Sephacryl S-100 column (Amersham-Pharmacia) was used for size-exclusion chromatography. Samples of less than 4 ml were loaded onto the column at 0.4 ml/min and eluted at 0.8 ml/min in 150 mM NaCl, 50 mM Tris pH 7.5. The elution profile was determined by calibration of the column by protein standards of known molecular mass. The column was calibrated with a mixture of ribonuclease A (molecular mass 13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and albumin (67 kDa) (Amersham-Pharmacia). One milliliter fractions were collected in the size range for dimeric inhibin and analyzed by immunoblotting.

Affinity chromatography

Affinity chromatography was utilized for activin A purification and when necessary in the final preparation for inhibin A. For inhibin A, 10 mg inhibin-specific
R1 antibody coupled to resin (HiTrap; Pharmacia, Piscataway, NJ, USA) was a gift of Dr Nigel Groome (Oxford-Brookes University, Oxford, UK). For activin A, the monoclonal antibody 3D9 (Genentech) was coupled to an Affi-gel 10 matrix (Bio-Rad Laboratories, Hercules, CA, USA) by aqueous coupling and packed into columns (Econo-columns; BioRad). Columns were equilibrated in 100 mM sodium bicarbonate/150 mM NaCl, pH 8.5. Samples were recirculated over the column, washed until the baseline was stable, and eluted by low pH (50 mM glycine, 1% Triton X-100, 150 mM NaCl, pH 2.5). One milliliter fractions containing the eluted proteins were collected and analyzed by silver staining and immunoblotting.

**Immunoblotting and silver stain analysis**

Samples were electrophoresed through non-reducing 12% SDS-PAGE, and for immunoblotting transferred onto nitrocellulose. The blots were incubated in 5% non-fat dry milk in Tris-buffered saline, 0.1% Tween-20 (TBS-T) for between 2 h and overnight. For detection of both ligands, a combination of a biotinylated monoclonal antibody 6H5 (Genentech) and a biotinylated polyclonal chicken antibody was used. Antibodies were diluted into the blocking buffer at various concentrations (2–5 µg/ml), and the blots were incubated with the primary antibody for 1 h. Blots were washed in TBS–T, incubated with streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) for 1 h, washed, and visualized by enhanced chemiluminescence (ECL; Amersham-Pharmacia). For detection of inhibin alpha, an alkaline phosphatase-conjugated R1 antibody was used and directly detected by chemiluminescence (Immun-Star Chemiluminescent Detection; Bio-Rad). For analysis of purity, proteins were visualized by silver staining of non-reducing SDS-PAGE gels. For silver staining, the SDS-PAGE gels were fixed in ethanol, rehydrated, incubated with a silver nitrate solution, and developed in a sodium thiosulfate solution. Gels were photographed on a light box using a hand-held camera system (Kodak, Rochester, NY, USA) or vacuum dried for storage.

**Inhibin ELISAs**

ELISAs for inhibin A were used to monitor inhibin production. The inhibin A assay used a chicken IgY polyclonal antibody as both the immobilized and detection antibody and detected total inhibin (Baly et al. 1993). The chicken antibody was coated onto 96-well microtiter plates (Nunc) and incubated overnight. A dilution of the sample was incubated for 3 h, detected using a colorimetric assay and read on a microplate reader (Bio-Tek Instruments, Frederick, MD, USA). The sample was quantified by comparison to known concentrations of recombinant human inhibin A.

**Amino acid sequencing**

Purified inhibin A was subject to sequencing to verify the identity of the product and to determine if the N-terminus of the α-subunit was intact. Purified protein (1-5 µg) was electrophoresed through a non-reducing 12% SDS-PAGE gel and transferred onto PVDF membrane (TransBlot; Bio–Rad). The protein band was stained and excised from the membrane. The protein was then sequenced at the Microchemistry Facility at Harvard University (Boston, MA, USA).

**Anterior pituitary bioassay**

Inhibin and activin bioactivities were measured by their ability to modulate release of FSH from cultured anterior
pituitary cells. The anterior pituitary bioassay was performed as previously described with modification (Wilfinger et al. 1984, Krummen & Baldwin 1988). All cell culture media were purchased from Life Technologies (Rockville, MD, USA) unless indicated. All animals were treated in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Sixty-day-old adult female Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were killed by CO₂ and decapitated according to the approved Northwestern University IACUC protocols. The anterior pituitaries were collected, washed in suspension modified MEM (S-MEM), quartered and digested with trypsin (0·4% trypsin, 0·2% BSA in S-MEM BSA). Following dissociation, pituitary cells were plated at a density of 2·5 × 10⁵ cells per well in a 24-well tissue culture plate format in alpha-modified MEM, 10% charcoal-stripped heat-inactivated fetal bovine serum, 1% antibiotics–antimycotics (Gibco). Cells were allowed to recover for 12 h, washed in growth medium, and treated with ligands. After 48 h, medium was recovered, centrifuged to remove any remaining cells, and analyzed by RIA for FSH by the RIA core facility at Northwestern University.

**p3TP-luciferase assay**

The ability of activin to stimulate luciferase activity was used to measure activin bio-potency as previously described (Chapman & Woodruff 2001). A plasmid containing the synthetic p3TP promoter upstream of the luciferase gene (Attisano et al. 1996) was transfected into TSA cells by lipid transfection (Lipofectamine; Gibco). The p3TP-luciferase plasmid was provided by the J Massague Laboratory, Memorial Sloan-Kettering Cancer Center, New York, NY, USA. After transfection, cells were allowed to recover for 12 h in DMEM, 10% fetal bovine serum. The medium was replaced with serum-free medium containing the purified protein. After 24 h, the medium was removed, cells were lysed in Triton X-10-containing buffer and assayed by the addition of luciferin. Light production was measured in a luminometer (EG&G Berthold, Wildbad, Germany). Unless indicated, the protein lots used in the assays were inhibin A NU-35–45, activin A NU–43–82. For immunoneutralization of activin A activity, a monoclonal antibody for activin A (a gift from R&D Systems, Minneapolis, MN, USA) was added to the cell culture medium coincident with the addition of activin A. For inhibin antagonism of activin, inhibin at various concentrations was added simultaneously with 3 ng/ml activin A.

**Results**

**Recombinant protein production in cells grown in bioreactors**

Recombinant cell lines producing human inhibin A and activin A secrete a complex mixture of higher molecular mass and mature forms of the proteins (Figs 1 and 2). These cells are capable of proper glycosylation, cleavage and secretion of both inhibin and activin. The inhibin A cell line was cultured in self-contained, small-scale bioreactors that utilize hollow fiber technology (Fig. 2). In the bioreactors, cells receive nutrients from continuously circulating media. Cells and secreted proteins are separated...
Figure 4  Purification series for inhibin A. Immunoblot analysis with the 6H5 monoclonal antibody for the purification of inhibin A from activin A-containing medium. (A) Ion-exchange chromatography shows mature activin A elutes earlier in the profile in fractions 20–85 but overlaps with 32–34 kDa inhibin A. (B) Hydrophobic interaction chromatography removes residual 28 kDa activin A in the earlier fractions 90–120. In some runs, however, activin A remained in the 32–34 kDa inhibin A fractions (see panel (C)). (C) Size-exclusion chromatography is shown for a purification series where mature inhibin and activin did not separate in the hydrophobic interaction chromatography. Fractions not containing the precursor proteins (14–23) were pooled and used in affinity chromatography. (D) Affinity chromatography. Mature inhibin A can be purified from contaminating activin A by affinity chromatography that uses an antibody selective for the α-subunit (R1). The fastest migrating band on the gel most likely represents non-glycosylated inhibin A.
from this medium by a membrane with a molecular mass cut-off of 10 000 Da. Therefore, metabolic waste products can diffuse out away from the cells, while larger proteins and the cells are retained. Glucose, oxygen and other nutrients freely diffuse. Cell number increases while the culture is being established but does not exceed 1:5 times the original seeded number. However, this does not correlate to the amount of inhibin produced as measured by ELISA (Fig. 2C and data not shown). The amount of total inhibin A (precursor and mature) measured by ELISAs specific for inhibin is shown in Fig. 2C. Measurement of the media shows that these cells can produce a maximum of approximately 900 μg/ml per day. For a typical 30 day run, 400 mg total inhibin can be produced. Of this total material, approximately 90% is precursor material leaving less than 40 mg of initial mature inhibin as starting material. The activin A cell line did not produce sufficient quantities of recombinant protein when cultured in a bioreactor. However, this cell line did respond well to culturing in triple-surfaced large-scale tissue culture flasks. The amount of activin produced was not measured but final purification values suggest that the initial levels were greater than 10 mg/l.

Inhibin A purification

The purification protocol developed for inhibin A is a modification of the original purification procedure developed at Genentech. A diagram of the procedure is shown in Fig. 3. The general procedure is a three-step purification. In step one, the medium is enriched for inhibin A through an ion-exchange column. In step two, inhibin A is separated from activin A by hydrophobic interaction chromatography. In step three, the mature inhibin A is removed from the precursor proteins by size-exclusion chromatography. In some preparations, the purification of inhibin through an affinity column was used if activin was not removed in the prior purification steps. Immunoblotting and silver staining of gels for the results of the inhibin A purification series is shown in Fig. 4. Purity as measured by silver staining is approximated to be 99% (see later Fig. 6B).

A representative immunoblot of the fractions obtained in the initial step ion-exchange chromatography is shown in Fig. 4A. Recombinant inhibin A elutes as a broad peak in combination with activin A and precursor proteins. Fractions enriched in the mature (32–34 kDa) inhibin A forms are pooled (Fig. 4A, fractions 90–120). This column is unable to fully separate inhibin from activin but can decrease the amount of CHO proteins and activin A present in the medium.

To separate inhibin from activin, differences in hydrophobicity of inhibin and activin dimers were exploited. Figure 4B shows the results from hydrophobic interaction chromatography. Immunoblot analysis of fractions from the second purification step reveals that activin A and the non-glycosylated inhibin A elute in an early peak from fractions 75–120 while the mono- and di-glycosylated forms of inhibin A and the majority of the precursor elute at later fractions.

A final separation of mature, dimeric inhibin A from the precursor proteins was needed. This was possible to achieve on a size-exclusion column. These results are shown in Fig. 4C. There is some overlap between elution peaks of the precursors and mature proteins such that some of the mature inhibin is lost. The amount of final material obtained from starting material has been variable.

If activin still remained in the final preparation, as determined by silver staining and immunoblotting, an affinity column for inhibin was employed. The results of this column purification are shown in Fig. 4D. The column is specific for inhibin isoforms, including precursor material. Final purity was determined by immunoblot and silver stain analysis (see later Fig. 6). The identity of the protein bands was determined to be inhibin A by microsequencing.

Activin A purification

Multiple monoclonal antibodies to activin A were available (Genentech). These were screened by immunoblotting against recombinant inhibin A media for those antibodies that only recognized mature activin A and not precursor forms or inhibin A (data not shown). Of the 24 antibodies that were screened, three were chosen as candidate antibodies for affinity purification of activin, and ultimately, monoclonal 3D9 was selected for its ability to bind to mature activin A but not inhibin A. The result of affinity chromatography for activin A is shown in a silver-stained blot and immunoblot (Fig. 5). The purity of the final preparation for both activin A and inhibin A was
estimated to be greater that 99% by silver staining of non-reducing SDS-PAGE gels for both proteins (Figs 4D and 6).

**Biological activity of the purified recombinant proteins**

To test the bioactivity of purified proteins, three assays were utilized. First, for activin A activity, TSA cells were transfected with an activin-responsive artificial promoter driving the expression of the luciferase gene (p3TP-luc) (Wrana et al. 1992, Chen & Johnson 1997). Transfected TSA cells respond to the purified activin A in a dose-dependent manner (Fig. 7A). The half-maximal stimulatory dose (EC_{50}) for the concentrations tested is 2 ng/ml. The stimulatory response to activin can be abolished by co-incubation of the cells with an antibody that neutralizes activin A (Fig. 7B). Secondly, inhibin is known to block activin stimulation of p3TP (Martens et al. 1997, Chapman & Woodruff 2001) and this was tested with the newly purified proteins. The addition of a 5-fold excess of inhibin A abolishes the activin A stimulation of p3TP. The third assay is the response of primary cultures of rat anterior pituitary cells to inhibin A and activin A (Fig. 8) (Vale et al. 1986). The addition of activin should stimulate FSH release while the treatment with inhibin should inhibit the response. A dose–response curve of a primary culture to the purified proteins is shown in Fig. 8. Both newly purified proteins elicit the expected biological response. The EC_{50} for the concentrations tested is 5 ng/ml activin A and 3 ng/ml inhibin A.

**Discussion**

The ability to understand the biological function of a protein often requires a pure source of that protein for experimental procedures. For members of the TGF-β superfamily, the ability to produce sufficient quantities that are biologically active has been difficult. Earlier work relied upon the purification from follicular and rete testes fluid (Ling et al. 1985, Robertson et al. 1986, Vale et al. 1986, Leversha et al. 1987, Vaughan et al. 1989). For
recombinant production of inhibin and activin, the production of one (inhibin) leads to the production of the antagonist protein (activin) (Mason et al. 1996). We established four criteria that must be met for production purposes. First, the subunits must be processed correctly from precursor to the mature 28–34 kDa size. Secondly, they must be assembled into homodimers of activin and heterodimers of inhibin. Thirdly, they must be secreted at high enough levels to warrant purification. Fourthly, the final products must be bioactive in established assays for inhibin A and activin A. These criteria exclude most conventional overexpression methods in E. coli and baculovirus (Cronin et al. 1998). However, we have developed two methods that meet these requirements and have led to the production of milligram quantities of bioactive recombinant human inhibin A and activin A.

For the inhibin A cell line, protein production was achieved using bioreactor technology. Culturing the inhibin A cell line in bioreactors can lead to production of media containing upwards of 900 µg/ml per day recombinant protein from approximately 6 × 10^7 cells. This level is necessary since the purification procedure for inhibin A causes significant losses. The activin A cell line, however, did not produce optimally in the bioreactors (data not shown). As an alternative, cells were grown in multilayer tissue culture flasks such that the density of cells per milliliter of media, and thus the concentration of ligand, could be tripled. Both culturing techniques yielded sufficient recombinant protein within 1 month of media collection to continue with the purification process.

Inhibin A was purified using a series of traditional chromatography columns. The lack of large quantities of...
an inhibin-specific antibody has made large-scale affinity purification difficult. An affinity column that contained the α-subunit-specific antibody was used only in the final preparation of inhibin A and only if the final preparation was determined to contain activin A. The drawback of this antibody is that it recognizes precursor forms as well as mature inhibin. Thus medium placed over the column must be devoid of most if not all precursor proteins in order to optimize the amount of mature inhibin purified. Newly isolated recombinant medium, however, contains an excess of precursor proteins over mature proteins. Therefore, the antibody column must be used following the removal of precursor proteins later in the purification process. In addition, the affinity of this antibody for the antigen is quite strong such that retrieval of inhibin is often difficult, requiring multiple elutions in low pH buffers with the addition of detergent. In some cases, denaturation of the column with guanidine HCl and refolding of the eluted protein was necessary (N Groome, unpublished observations).

In lieu of an affinity-based approach, the biochemical purification of inhibin often succeeds with sufficient yields of recombinant protein. Three or four column chromatography steps are necessary. Elution of the desired protein was monitored by immunoblot analysis following each of the columns. In the first step, a strong cation-exchange resin is used to remove a significant quantity of contaminating endogenous secreted proteins. This ion-exchange chromatography exploits the charge differences in inhibin A and activin A, which have different theoretical isoelectric points (approximately 8·2 and 7·6 for precursor and mature inhibin A respectively, vs 8·3 and 7·1 for precursor and mature activin A respectively). The ligands are bound to the column at a pH of 6·0 where the net charges are positive and eluted at a pH of 7·5 where the net charge on activin A becomes negative. In several instances, all of the activin A eluted earlier in the profile separable from inhibin. This simplified the purification process since the next hydrophobic interaction chromatography step could be eliminated.

Hydrophobic interaction chromatography was used to successfully isolate inhibin from activin A. This is probably due to the strongly hydrophobic N-terminus of the α-subunit. Use of a phenyl–Sepharose resin exploits the differences in hydrophobicity of the mature as well as the precursor proteins. A gradient of decreasing ionic strength was used to elute the column. Activin and non-glycosylated inhibin elute earliest. These fractions are easily isolated from the fractions that contain mature, dimeric, glycosylated inhibin A that elutes later in the elution.

Size-exclusion chromatography was used to separate precursor from mature proteins. Although the elution profiles overlapped for several fractions, enough mature 32–34 kDa inhibin was isolated from the precursors. The elution of 32–34 kDa inhibin A with higher molecular mass forms may suggest that the dimeric mature proteins can complex with dimeric precursors. In addition, in several purification runs, activin still contaminated the final preparations. In most cases, this seemed to be due to overloading of the previous columns. A small affinity column containing the inhibin-specific R1 antibody was then used to isolate inhibin from activin therefore guaranteeing the final purity of the protein preparation.

Activin A was purified through affinity chromatography by using an antibody that only recognized mature 28 kDa activin A. This was possible since screening monoclonal antibodies against secreted recombinant inhibin A media revealed one antibody, 3D9, that recognized mature activin, and not precursor proteins or inhibin A. It is possible that other antibodies that have similar properties will be needed in the future since additional sources of 3D9 are unavailable. Screening of antibodies for an activin B-specific antibody for affinity chromatography also could be used to develop an activin B purification method.

Finally, any purified protein must maintain all biological activity of the physiologically produced protein therefore making bioassays critically important. For the inhibins and activins, the physiological role (the regulation of pituitary FSH release) can be mimicked in tissue culture cells, thus providing an excellent test of the potency of the purified proteins (Vale et al. 1986). Anterior pituitary cells respond appropriately to the human recombinant ligands as measured by RIA for FSH in a dose that is similar to those previously reported for activin A (Vale et al. 1986, Schwall et al. 1988). Human recombinant inhibin A was less potent in inhibiting FSH release from primary rat anterior pituitary (EC50 approximately 3 ng/ml) than that reported for porcine inhibin A isolated from follicular fluid (0·5 ng/ml) (Vale et al. 1986) but similar to the EC50 (2·7 ng/ml) for human recombinant inhibin A purified by Mason et al. (1996). This may reflect a species difference in this particular assay for human and porcine inhibin as the α-subunit is not identical. A second assay, the activation of an activin-responsive promoter, was also employed for monitoring activin activity as well as inhibin antagonism of that response. The p3TP promoter contains three AP-1 sites and the plasminogen activator inhibitor–1 promoter and is activin-responsive (Wrana et al. 1992, Chen & Johnson 1997). TSA cells transfected with this reporter respond in a dose-dependent manner to the purified protein. The activation for the dose range tested has an EC50 of 2 ng/ml, a concentration quite similar to other assays for both human and porcine activin function (Vale et al. 1986, Schwall et al. 1988, Mason et al. 1989, Phillips et al. 1999). This activation can be blocked by the co-stimulation of inhibin A as has been reported (Martens et al. 1997, Chapman & Woodruff 2001).

In conclusion, by using a mammalian cell expression system and a biochemical purification approach it was possible to purify sufficient quantities of biologically active inhibins and activins for research purposes. It is likely that
similar approaches can be employed to purify the other isoform of inhibin and activin, including inhibin B and activin B.

Acknowledgements

The authors wish to thank Drs Jennie Matber, Roger Pai, Diana Stoks and Robert Shawley at Genentech Inc. (South San Francisco, CA, USA) for the original purification protocol for inhibin A, the inhibin and activin cell lines, and the inhibin A and activin A antibodies, as well as thanking Roger Pai for his helpful advice. Thanks to Dr Nigel Groome (Oxford-Brookes University, Oxford, UK) as thanking Roger Pai for his helpful advice. Thanks to Dr Nigel Groome (Oxford-Brookes University, Oxford, UK) for the R1 affinity column, and to Monica Tsang at R&D Systems (Minneapolis, MN, USA) for the neutralizing antibody to activin A. Thanks also to Magdalena Suszko for the dose–response curve of activin A in rat anterior pituitary cells, Bridget Mann and the RIA Core Facility at Northwestern University for the RIA assays, and the J Massague Laboratory (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) for the p3TP-luciferase reporter plasmid. This study was supported by NIH grants HD-3796 and HD-28048 to T K W. S A P is a Fellow of the Northwestern University Program in Endocrinology, Diabetes and Hormone Action (DK07169).

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Received 10 August 2001
Accepted 17 September 2001