Purification of an Equine Apotransferrin Variant (Thyromedin) Essential for Thyroid Hormone Dependent Growth of GH_1 Rat Pituitary Tumor Cells in Chemically Defined Culture†

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ABSTRACT: Pituitary tumor cells require thyroid hormones for growth in vivo [Sorrentino, J. M., Kirkland, W. L., & Sirbasku, D. A. (1976) *J. Natl. Cancer Inst. 56*, 1155–1158]. In vitro, GH_1 rat pituitary tumor cells were studied in a serum-free defined medium (PCM-10) formulated with Ham's F12 and Dulbecco's modified Eagle's media (1:1, v/v) supplemented with 2.2 g/L sodium bicarbonate, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 10 μg/mL human transferrin, 50 μM ethanolamine, 10 μg/mL insulin, 10 ng/mL selenous acid, 0.1 nM 3,5,3′-triiodothyronine (T_3), and 500 μg/mL bovine serum albumin and in the same medium without T_3 (PCM-0). The cells only grew in PCM-10 when low concentrations of horse serum were added. Attempts to replace the serum factor requirement with known growth factors and adhesion proteins were unsuccessful. The M_r 65 000–72 000 serum factor regulating T_3-induced growth (thyromedin) was purified to homogeneity and identified as equine transferrin R and/or D by amino acid sequencing. The ED_{50} in PCM-10 was 17–40 μg/mL (260–620 nM) while in PCM-0 half-maximum growth was not achieved at 200 μg/mL. Concentrations of 75 μg/mL in PCM-10 caused 80% of serum-stimulated growth rate. Removal of iron from thyromedin, and assay in iron salts reduced PCM-10, increased the specific activity 110–270-fold to ED_{50} 150 ng/mL (2.3 nM); at 1.0 μg/mL, growth in PCM-10 was 16-fold greater than in PCM-0. Iron saturation of thyromedin caused total loss of biological activity. We conclude that the horse transferrin variant isolated in this report is active as apotransferrin.

Thyroid hormones are required for growth rat pituitary tumor cells and normal rat somatotrophs in vitro (Hinkle & Kinsella, 1986; Kirkland et al., 1976; Miller et al., 1987; Samuels et al., 1973, 1974) and in vivo (Astier et al., 1980; DeFesi et al., 1979; Sorrentino et al., 1976b; Surks & DeFesi, 1977). In particular, the GH_1 rat pituitary cell line has been useful because thyroid hormones regulated both growth (Samuels et al., 1973) and the expression of the growth hormone gene (Evans et al., 1982; Kumari-Siri & Surks, 1985; Samuels & Shapiro, 1976; Tsai & Samuels, 1974). The other clonally derived lines often studied included the GH_3 (Tashjian et al., 1968), the GC cells which originated from the GH_3 population (Bancroft & Tashjian, 1971), the GH_4C_1 cells derived from the GH_3 line (Tashjian et al., 1970), and the GH_3C_{14} cells obtained also from the GH_3 population (Kirkland et al., 1976; Sorrentino et al., 1976a,b).

Hayashi and Sato (1976) were first to grow GH_3 cells in a completely serum-free medium containing 3,5,3′-triiodothyronine (T_3),[1] partially purified IGF-I, transferrin, thyrotropin-releasing hormone, and parathyroid hormone. Later, insulin and IGF-I were shown to be the most important components of the medium (Bottenstein et al., 1979; Hayashi, 1984; Hayashi et al., 1978).

Our attempts to grow rat pituitary tumor cells in defined medium revealed many problems (Riss et al., 1986, 1989; Stewart & Sirbasku, 1987, 1988a,b). First, <10% of the cells survived passage from serum-containing culture into defined medium. Second, the effects of polypeptide mitogens were unlike those reported by Bottenstein et al. (1979), Hayashi (1984), Hayashi and Sato (1976), and Hayashi et al. (1978). Deletion of insulin and IGF-I had no effect on growth; aFGF and bFGF were growth inhibitors. Third, the GH_1 cells did not grow in any defined medium even with T_3 and insulin. Fourth, the GH_3 and GH_4C_1 lines grew only in defined medium containing supraphysiological concentrations of T_3 and rapidly progressed to hormone autonomy. Because rat pituitary tumor cell lines were grown in serum-containing medium without losing T_3 responsiveness (Kirkland et al., 1976; Riss et al., 1989; Samuels et al., 1973; Stewart & Sirbasku, 1987, 1988a,b), we concluded that an unidentified component of serum (i.e., thyromedin) was required for hormone dependence (Stewart & Sirbasku, 1987, 1988a,b).

Using GH_1 cells in serum-free medium, we purified an M_r 65 000–72 000 thyromedin from horse serum and identified at as the R and/or D variant of equine transferrin. This

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[1] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CPD, cell population doubling(s); DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; F12–DME, 1:1 (v/v) mixture of Ham's F12 and Dulbecco's modified Eagle's medium containing 15 mM HEPES and 2.2 g/L sodium bicarbonate (pH 7.2); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; PBS, Dulbecco's phosphate-buffered saline, pH 7.2; PDGF, platelet-derived growth factor; *pI*, isoelectric point; RP-FPLC, reverse-phase fast-performance liquid chromatography; RP-HPLC, reverse-phase high-pressure liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Se, selenous acid; TFA, trifluoroacetic acid; TGFβ, transforming growth factor type β; T_3, 3,5,3′-triiodothyronine.

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variant was essential for thyroid hormone dependent pituitary cell growth in defined medium. In other experiments, the iron content of the variant proved critical to the biological activity. Depletion of iron in thyromedin, and assay in iron salts reduced medium, increased specific activity more than 100-fold. Iron saturation of thyromedin, and assay in iron salts containing medium, caused total loss of activity. The data presented indicate that the variant(s) isolated was (were) biologically active as apotransferrin.

Experimental Procedures

Materials. Powdered medium, fetal bovine serum, and horse serum were purchased from GIBCO. Standard F12-DME contained 0.05 mg/L $Fe(NO_3)_3 \cdot 9H_2O$ and 0.417 mg/L $FeSO_4 \cdot 7H_2O$. The total iron content of the dissolved medium was 65 ng/mL or 64% of the expected amount (Atomic Absorption Spectroscopy, Galbraith Laboratories, Knoxville, TN). Unless otherwise stated, the medium used was this iron salts containing formulation. The same medium without the iron salts was purchased from GIBCO; total iron content was <10 ng/mL. When used in assays, this medium was designated "iron salts reduced".

A mixture of insulin, human transferrin and Se (ITS), and mouse EGF was purchased from Collaborative Research. Human recombinant IGF-I and IGF-II were gifts from Dr. B. Daniel Burleigh (IMC Pitman-Moore, Terre Haute, IN). Porcine PDGF and TGFβ were from R & D Systems, and human recombinant bFGF was from Amgen. Laminin was from GIBCO and chondronectin from Bethesda Research Laboratories. Serum spreading factor/vitronectin was a gift from Dr. David Barnes, Oregon State University. Bovine plasma fibronectin, powdered Dulbecco's PBS, BSA, Etn, T_3, and Trizma base were purchased from Sigma. Trypsin/EDTA in Hank's balanced salt solution without calcium and magnesium salts was purchased from Irvine Scientific. Crude porcine pancreatic trypsin was purchased from ICN. Affi-Gel Blue resin (100–200 mesh), Sepharose 6B, concanavalin A–Sepharose 4B, DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B, the RP-FPLC ProRPC C_1C_8 HR 5/10 column (average pore size 300 Å), polybuffer 74, and polybuffer exchanger (PBE 94) were purchased from Pharmacia. The Vydac C_4 RP-HPLC column (4.6 mm × 250 mm, 5-μm average pore size) was purchased from Rainin. The anion-exchange resin AG 1-X 8 (100–200 mesh) was purchased from Bio-Rad. Acetonitrile and ChomAR-HPLC-grade distilled water were from Mallinckrodt. High-purity TFA, reagent-grade ammonium sulfate, and sodium chloride were purchased from Fisher. Dialysis membranes (Spectrapor 3, molecular weight cutoff 3500) were from Spectrum Medical Industries.

Water and Stock Solution Preparations. With the exception of RP-FPLC and RP-HPLC, all water used in this study was purified as described (Riss et al., 1986). The total iron content was <10 ng/mL (atomic absorption spectroscopy).

The following stock solutions were prepared and stored at −20 °C in aliquots sufficient for use within a few months: BSA, 60 mg/mL in saline; Etn, 10 mM in water; and T_3, 0.10 μM in 0.01 N sodium hydroxide. The ITS mixture was diluted to 10 mg/mL insulin, 10 mg/mL transferrin, and 10 μg/mL Se. Growth factors were dissolved in the sterile solutions recommended by the suppliers; the mitogenic activity of each was confirmed by using serum-free defined medium cell culture bioassays (Karey & Sirbasku, 1988; Ogasawara & Sirbasku, 1988; Riss & Sirbasku, 1987; Riss et al., 1986, 1988). The adhesion proteins preparations were assayed with MCF-7 human breast cancer cells in serum-free defined medium (Karey & Sirbasku, 1988).

Stock Cell Culture. The GH_1 cell line was obtained from American Type Culture Collection. The cultures were free of mycoplasma contamination throughout this study (Myco-Tect System, GIBCO). Stock cultures were maintained in 100-mm-diameter dishes incubated in a 5% (v/v) CO_2 and 95% (v/v) air-humidified atmosphere at 37 °C in 15 mL of F12–DME supplemented with 15 mM HEPES, pH 7.2, 2.2 g/L sodium bicarbonate, 12.5% (v/v) horse serum, and 2.5% (v/v) fetal bovine serum. The cells were passaged at 4.0×10^5/dish at 3–4-day intervals by treatment at room temperature with 1.5 mL of trypsin/EDTA in Hank's balanced salt solution. The action of trypsin was stopped after 2–4 min by addition of 10 mL of medium containing 15% serum; the cells were harvested by centrifugation at 300–500*g*, suspended in fresh F12–DME containing serum, and dispensed to new dishes. The procedure was modified when the cells were to be used in serum-free assays. After centrifugation, the cells were suspended in 10-mL portions of F12–DME and washed 2–3 times with serum-free medium.

Cell Number Bioassay of Thyromedin Activity. Only 3–4-day-old stock cultures were used in cell number assays. Washed cells were seeded at $(2–4) \times 10^4$/35-mm diameter dish containing 3.0 mL of F12–DME supplemented with 15 mM HEPES (pH 7.2), 2.2 g/L sodium bicarbonate, 10 μg/mL insulin, 10 μg/mL human transferrin, 10 ng/mL Se, 500 μg/mL BSA, 0.1 nM T_3, and 10 μM Etn (PCM-10) or into the same medium prepared without T_3 (PCM-0). Whole serum or aliquots from chromatographic column fractions were added in volumes ≤100 μL. After 6–7 days at 37 °C, 0.2 mL of 2% (w/v) crude pancreatic trypsin in Dulbecco's PBS containing 152 mM EDTA was added to each dish. The dishes were incubated at room temperature for 2 min before addition of 0.4 mL of horse serum. When the dishes contained serum, the medium was aspirated and replaced with F12–DME before adding trypsin. The contents of each dish were diluted to 10 mL with Isoton II (Coulter Electronics) and cell numbers (i.e., CPD) determined by using a Coulter counter Model ZBI. Duplicate or triplicate plates were used for each determination. Triplicate dishes were harvested for counting within 1–2 h of seeding to determine initial cell numbers for calculation of CPD.

Thyromedin specific activity (ED_{50}) was half the difference between the average cell number measured on day zero and those measured with saturating thyromedin concentrations in PCM-10 at the end of the experiments. One unit of thyromedin activity was defined as the amount required to achieve ED_{50}.

Estimation of Molecular Weight and Homogeneity by SDS–PAGE. Analysis by SDS–PAGE was done by the method of Laemmli (1970) using 0.75-mm-thickness 5% (w/v) stacking and 10% (w/v) acrylamide resolving gels. Electrophoresis was performed at 200 V for 45 min followed by staining with 0.2% (w/v) Coomassie Brilliant Blue dye and destaining with 40% (v/v) methanol and 10% (v/v) acetic acid. Silver reagent staining was done after removing the Coomassie dye using procedures described in a kit purchased from Bio-Rad. The prestained proteins used to estimate molecular weight were myosin H chain (199 000), phosphorylase *b* (104 000), BSA (66 000), ovalbumin (42 000), α-chymotrypsinogen (25 000), β-lactoglobulin (18 000), and lysozyme (15 000) purchased from Bethesda Research Laboratories.

Protein Sequencing and Amino Acid Composition. Samples from concanavalin A–Sepharose were analyzed by automated Edman degradation using the Beckman Model 890 M automatic sequencer (Chin et al., 1981). Amino acid sequence

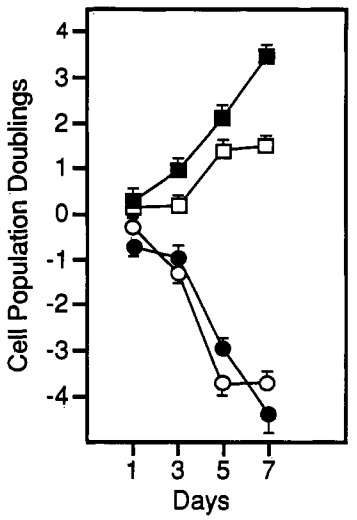

FIGURE 1: Growth of GH_1 cells in PCM-0 and PCM-9 ± serum supplementation. Growth rates are shown as follows: closed circles, PCM-0; open circles, PCM-9; open squares, PCM-0 containing 5% (v/v) serum from thyroidectomized rats; closed squares, PCM-9 containing 5% (v/v) serum from thyroidectomized rats. Data are averages of triplicate dishes (±SD, bars) harvested on the designated days. Serum was from adult female thyroidectomized Sprague-Dawley rats 7 days after surgery.

analysis of the ProRPC C_1C_8 RP-FPLC and Vydac C_4 RP-HPLC samples was done by the micromethods of Hunkapiller et al. (1983), using an Applied Biosystems 477A instrument.

Other samples were subjected to 6 N hydrochloric acid hydrolysis for 21 h at 110 °C and thereafter applied to a Durrum-500 amino acid analyzer to determine the amino acid composition (Chin et al., 1981).

Results

Serum Factor Requirement for T_3-Responsive Growth. Growth of the GH_1 cells in PCM-0 is shown in Figure 1. Within 24 h after transfer from serum-containing medium, the cells began to decrease in number and continued to do so throughout the incubation. By day 7, ≤20% of the cells were viable. Addition of 1.0 nM T_3 to the PCM-0 (to give PCM-9) had only a small effect on the maintenance of cell numbers (Figure 1). In contrast, addition of 5% (v/v) serum from thyroidectomized rats to PCM-0 increased cell survival and promoted 1.6 CPD/7 days above seed density levels (Figure 1). In replicate experiments, this growth varied between 0.5 and 2.0 CPD/7 days. Addition of the same concentration of thyroidectomized serum to PCM-9 resulted in 3.5 CPD/7 days (Figure 1), giving a population doubling time of 48 h, which was 90% of the growth rate of horse/fetal bovine serum containing cultures (data not shown).

Attempts To Substitute Growth Factors and Adhesion Proteins for Thyromedin Activity. Polypeptide growth factors of five functional families [see volumes in Barnes and Sirbasku (1987a,b)] were examined for the ability to improve GH_1 cell survival and/or growth in PCM-0, PCM-9, and PCM-10. The following factors were assayed at the designated concentrations: IGF-I, 0.1–20 ng/mL; IGF-II, 0.1–20 ng/mL; bFGF, 0.05–10 ng/mL; aFGF, 1.0–20 ng/mL; EGF, 0.05–20 ng/mL; TGFβ, 0.1–20 ng/mL; PDGF, 0.1–10 ng/mL. None increased survival or promoted growth. Also, thyrotropin-releasing hormone and parathyroid hormone had no effects.

EGF, bFGF, and aFGF were inhibitory under all conditions tested. This was expected because GH_4C_1 cells growing in response to supraphysiological concentrations of T_3 were inhibited by 10–20 ng/mL bFGF or EGF (Riss et al., 1986, 1989) as were GH_3 cells by EGF in serum-containing culture (Johnson et al., 1980; Schonbrunn et al., 1980).

IGF-I and IGF-II had no effect on survival or growth of GH_1 cells in defined medium. Presumably, the lack of IGF-I response in PCM-10 was due to the presence of a supraphysiological concentration of insulin (i.e., 10 μg/mL) known to saturate the type I $\alpha_2\beta_2$ IGF-I receptor (Massague & Czech, 1982). To test this, insulin was removed from PCM-0 or PCM-10; experiments ± IGF-I and IGF-II in the modified media showed no growth effects.

In other studies, the adhesion proteins fibronectin (Hynes & Yamada, 1982), serum spreading factor/vitronectin (Barnes & Silnutzer, 1983; Hayman et al., 1983), laminin (Timpl et al., 1979), and chondronectin (Hewitt et al., 1980) did not improve survival in PCM-10 at concentrations of 2–20 μg/mL.

Purification of Horse Serum Thyromedin. A larger scale thyromedin source than rat serum was sought for purification. In experiments not presented, thyroid hormone depleted horse serum prepared by the methods of Samuels et al. (1979) and thyroidectomized rat serum gave the same magnitude of GH_1 growth response to T_3. Also, GH_1 cells have been grown for several years in a medium containing horse serum (Tashjian et al., 1968) and have maintained T_3 responsiveness (Stewart & Sirbasku, 1987, 1988a,b). Unless otherwise noted, the isolation steps were conducted at 4–7 °C. Table I summarizes the quantification of the purification. Protein concentrations were estimated by using a kit purchased from Bio-Rad and BSA as standard (Bradford, 1976).

Table I: Summary of the Purification of Horse Serum Thyromedin

step	total protein (mg)	% protein yield	ED_{50} (μg/mL)	units of act.	% act. yield
horse serum	30000	100	1400	21400	100
ammonium sulfate (50–65%)	9000	30	1000	9000	42
Affi-Gel Blue	670	2.2	500	1340	6.3
DEAE-Sepharose	224	0.74	100	2240	10.5
Sepharose 6B	53.3	0.18	80	668	3.1
concanavalin A–Sepharose	50.1	0.17	40	1250	5.8
phenyl-Sepharose	20.1	0.07	22	912	4.3

Step 1. For the purification, solid ammonium sulfate (156.5 g) was added over 30 min to 500 mL of horse serum (i.e., 50% saturation). The solution was stirred for 2 h; the precipitate was removed by centrifugation at 11900*g* for 30 min and discarded. The supernatant (470 mL) was treated with an additional 47.5 g of solid ammonium sulfate over 15 min to raise the salt concentration to 65% saturation. After an additional 2 h of stirring, the precipitate was collected as before, dissolved in approximately 200 mL of PBS, and dialyzed twice against 4 L of the same buffer. The bioactivity in this fraction was assayed only in PCM-10. This solution contained 30% of the protein and 42% of the units of activity in 500 mL of horse serum (Table I).

Step 2. Affi-Gel Blue chromatography was done at room temperature. The resin was equilibrated with PBS, pH 7.2, and the ammonium sulfate precipitate (500 mL) mixed with 600 mL of resin. Materials not binding to Affi-Gel Blue were collected by filtration. Protein bound to the resin was eluted with 500 mL of PBS containing 1.0 M sodium chloride. The resin was then washed with 2.0 L of PBS and the flow-through fraction mixed again with the resin. The associated protein was separated as before. The high salt eluted material was biologically inactive in PCM-10 (data not shown). The PBS-eluted fraction contained 2.2% of the protein in 500 mL of hose serum and 6.3% of the units of activity (Table I).

Step 3. A DEAE-Sepharose CL-6B column (5.0 cm × 45

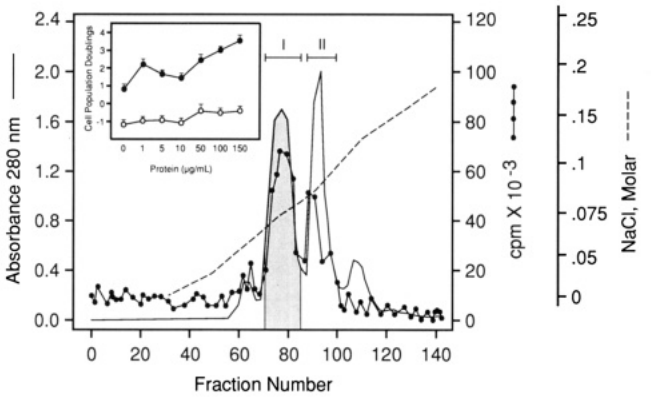

FIGURE 2: DEAE-Sepharose CL-6B ion-exchange chromatography of the thyromedin preparation from the Affi-Gel Blue step. The conditions of the chromatography, sample preparation, and the [^{3}H]TdR incorporation assay (cpm) are described in the text. The bars indicate the fractions combined to form pools I and II. The insert shows the cell number assays with pooled I fractions (shaded area) in PCM-0 (open circles) and PCM-10 (closed circles). The data are the averages of triplicate dishes (±SD, bars) after 6 days.

cm) was equilibrated with 0.10 M Tris-HCl, pH 8.6. The sample (550 mL) from *step 2* was dialyzed twice against 4 L of the equilibration buffer and the pH adjusted to 8.6 with 1.0 N HCl before application to the column at a rate of 1.0 mL/min. No A_{280nm} material eluted in the 1.0-L wash with equilibration buffer.

The column was eluted with a 3.6-L total volume linear gradient of equilibration buffer alone and buffer containing 0.4 M sodium chloride at a flow rate of 0.8 mL/min, collecting 20-mL fractions; the A_{280nm} elution profile is shown in Figure 2. The conductance of selected fractions was measured, and even-numbered fractions (20 μL) were assayed in PCM-10 for thyromedin activity by a [*methyl*-^{3}H]thymidine incorporation method (Riss et al., 1986).

Two major A_{280nm} peaks were identified (Figure 2). Pool I material showed 10 times more activity in PCM-10 than in PCM-0. Pool II material showed little difference in activity between PCM-10 and PCM-0 and was overall one-tenth as potent (data not shown).

The ED_{50} of pool I was estimated at 100 μg/mL by the cell number method (insert, Figure 2). Significant growth was observed in PCM-10 at 1–5 μg/mL while none was found in PCM-0 at protein concentrations of up to 150 μg/mL. DEAE-Sepharose pool I had 10.5% of the total units of activity present in 500 mL of horse serum and 0.74% of the protein (Table I).

Step 4. Pool I (220 mL) from DEAE-Sepharose CL-6B was dialyzed against 4 L of PBS followed by concentration to 7 mL using Amicon ultrafiltration with a YM-10 membrane and nitrogen gas pressure. The final sample was divided equally and each portion applied to a Sepharose 6B column (1.5 cm × 115 cm) equilibrated with PBS. Fractions (3.5 mL) were collected at a flow rate of 0.2 mL/min. The A_{280nm} elution profile of the column showed two major peaks (Figure 3). Selected fractions were assayed for mitogenic activity in PCM-10 by the [*methyl*-^{3}H]thymidine incorporation method. In experiments not presented, the activities in the two peaks were compared in PCM-0 and PCM-10 by using the [^{3}H]TdR incorporation assay. The first showed no activity in either PCM-0 or PCM-10 while the second showed biological activity in PCM-10 but not PCM-0. The active fractions were pooled (shaded area, Figure 3) and assayed by the cell number method in PCM-0 and PCM-10 to obtain an ED_{50} of 80 μg/mL (insert Figure 3).

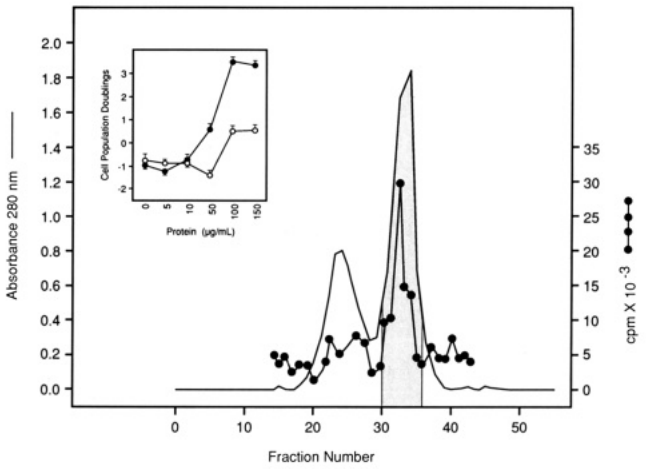

FIGURE 3: Sepharose 6B molecular sieve chromatography of half of the thyromedin preparation from the DEAE-Sepharose step. The conditions of the chromatography, sample preparation, and the [^{3}H]TdR incorporation assay (cpm) are described in the text. The insert shows a cell number assay with the pooled (shaded area) fractions in PCM-0 (open circles) and PCM-10 (closed circles). The data are the averages of triplicate dishes (±SD, bars) after 6 days.

The second half of the DEAE-Sepharose sample was applied to the Sepharose 6B column under identical conditions. The active fractions, pooled from both columns, contained 3.1% of the units of activity in horse serum and 0.18% of the protein (Table I).

Step 5. The Sepharose 6B active pool was concentrated by Amicon ultrafiltration as described above. The buffer was changed to 0.010 M Tris-HCl (pH 7.5) containing 0.15 M sodium chloride by three cycles of 10-fold concentrations with 50-mL aliquots. The sample (4.5 mL containing 53.3 mg of protein) was divided equally and each applied to a concanavalin A–Sepharose 4B column (2.5 cm × 20 cm) at a flow rate of 0.2 mL/min, collecting 4.0-mL fractions. The column was washed with several volumes of the Tris/sodium chloride buffer. Less than 5% of the protein eluted in the wash and contained no thyromedin activity.

The protein associated with concanavalin A was eluted with 500 mM D-glucose; a single A_{280nm} peak was obtained (Figure 4). These fractions were pooled (shaded area, Figure 4). The second half of the Sepharose 6B fraction was carried through the same procedure. The pooled material from both columns (40 mL) was active in PCM-10 but not in PCM-0 and gave an ED_{50} of 40 μg/mL (Table I). This fraction contained 0.17% of the protein in 500 mL of horse serum and 5.8% of the units of activity.

Step 6. The buffer of the concanavalin A–Sepharose 4B active pool was changed to 0.10 M sodium phosphate, pH 7.0, using 50-mL volumes in three 10-fold concentrations with the Amicon ultrafilter. The 7.5 mL was divided equally and brought to 3.0 M sodium chloride by addition of solid salt. These solutions were applied separately at room temperature to a phenyl-Sepharose column (2.5 cm × 30 cm) equilibrated with 0.10 M sodium phosphate (pH 7.0) containing the high salt concentration. The column was eluted at 0.2 mL/min (2.0-mL fractions) with a linear gradient (300-mL total volume) of 0.10 M sodium phosphate, pH 7.0, with 3.0 M sodium chloride and the same buffer with no salt.

A minor A_{280nm} component eluted early in the gradient. The major peak eluted between 2.2 and 1.8 M sodium chloride (Figure 5). The active fractions (shaded area, Figure 5) were pooled, dialyzed/concentrated into PBS with the Amicon ultrafilter, sterilized and stored at 4 °C. The combined pools from two columns contained 20 mg of protein which was 0.07%

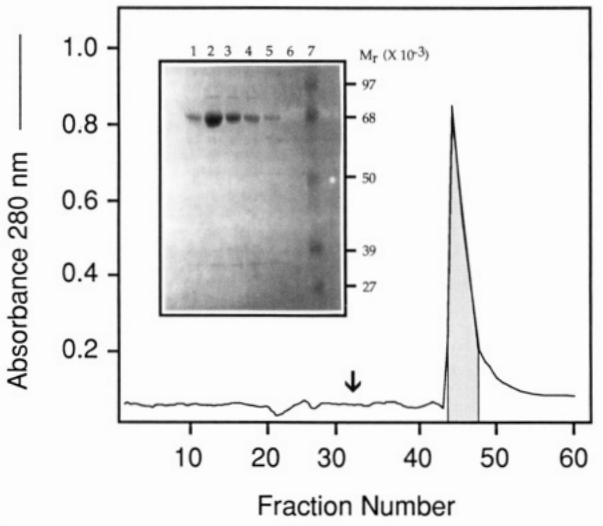

FIGURE 4: Concanavalin A–Sepharose affinity chromatography of half of the thyromedin preparation from two pooled Sepharose 6B steps. The conditions of the chromatography and sample preparation are described in the text. The arrow shows the start of elution with 500 mM D-glucose in equilibration buffer. The insert shows SDS–PAGE with Coomassie Blue staining of constant-volume aliquots from fractions 44–48 (lanes 1–5) and fraction 55 (lane 6). Lane 7 shows the migration of molecular weight standards. The shaded area was pooled for bioassay.

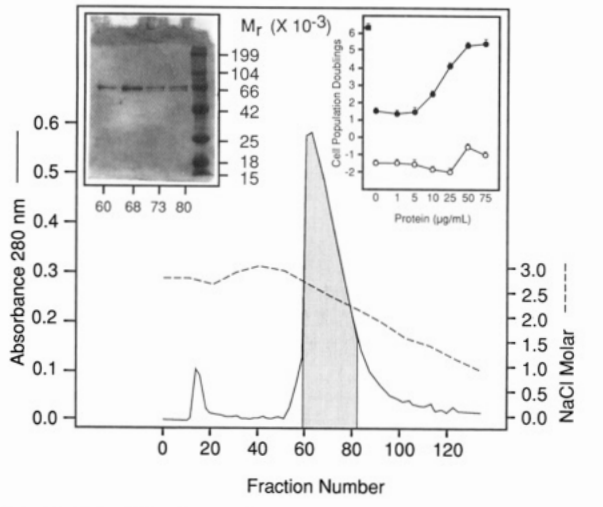

FIGURE 5: Phenyl-Sepharose hydrophobic interaction chromatography of half of the thyromedin preparation from two pooled concanavalin A–Sepharose steps. The conditions of the chromatography are described in the text. The shaded area was pooled and dialyzed/concentrated into PBS before assay of thyromedin activity. The insert on the left shows an SDS–PAGE and Coomassie Blue dye analysis of constant-volume aliquots of the designated fractions (lanes 1–4) while lane 5 shows the migration of molecular weight markers. The insert on the right shows the cell number assay in PCM-0 (open circles) and PCM-10 (closed circles) in response to thyromedin addition. The shaded square shows GH_1 cell growth in PCM-0 with 5% horse serum. The data are the averages of triplicate dishes (±SD, bars) after 6 days.

of that originally present in 500 mL of horse serum. Replicate preparations have yielded 5–20 mg of thyromedin.

Bioassays in PCM-0 and PCM-10 are shown by the right side insert in Figure 5. Thyromedin at 75 μg/mL in PCM-0 increased cell survival to seed density levels. In PCM-10, 50–75 μg/mL saturated growth at 80% of the rate promoted by horse serum. Thyromedin showed an ED_{50} of 22 μg/mL (340 nM).

From the final activity yield of 4.3%, and the total protein isolated (20 mg), the maximum calculated concentration of

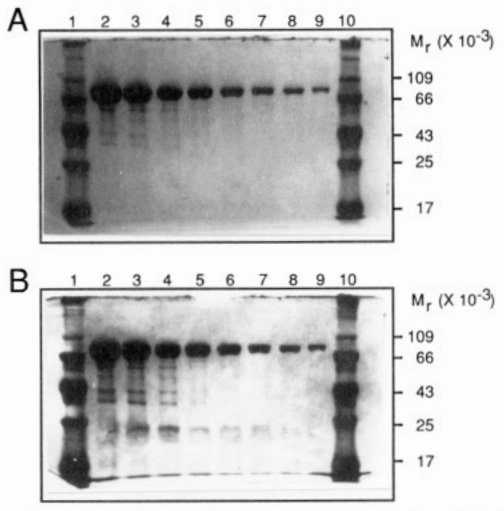

FIGURE 6: SDS–PAGE analysis of thyromedin from phenyl-Sepharose. (A) Coomassie Blue staining of the gel. Lanes 1 and 10 show the standard molecular weight proteins. Lanes 2–9 received 24, 12, 6, 3, 1.5, 0.75, 0.375, and 0.187 μg of thyromedin, respectively. (B) Silver staining of the gel shown in (A).

thyromedin originally present in horse serum was 930 μg/mL which was 1.5% of the total.

SDS–PAGE Estimation of Molecular Weight and Homogeneity of Thyromedin at Various Steps of Purification. Beginning with the concanavalin A–Sepharose elution, a single major A_{280nm} peak coincided with thyromedin activity, implying a high degree of homogeneity. SDS–PAGE was done to further analyzed the preparation.

Figure 4 shows a Coomassie Blue stained SDS–PAGE analysis of selected fractions from concanavalin A–Sepharose. A major band of M_r 65 000 was found as were components of M_r 75 000 and 32 000.

A similar SDS–PAGE analysis of individual active fractions from phenyl-Sepharose showed a characteristic pattern seen clearly only when ≥0.1-μg amounts of protein were analyzed. Figure 5 shows a single band of M_r 66 000 obtained from a fraction from the leading edge of the major A_{280nm} peak. Fractions with the maximum A_{280nm}, or those later in the peak, showed a doublet of M_r 66 000 and 64 000. The lower molecular weight form was less abundant. Previous studies of equine transferrin variants R and D described a similar doublet pattern (Chung & McKenzie, 1985).

The pooled activity from phenyl-Sepharose also was analyzed by SDS–PAGE and Coomassie Blue dye staining using greater amounts of protein. Application of 24, 12, and 6 μg per lane showed the presence of a major M_r 72 000 band (Figure 6A). Several lower molecular weight bands also were observed; their intensities increased during storage at 4 °C. As shown (Figure 6A), analysis of 187 ng of thyromedin again revealed the doublet band pattern.

When the silver staining method was repeated with the acrylamide gel shown in Figure 6A, the lanes receiving 24, 12, and 6 μg showed the major M_r 70 000 protein as well as the same lower molecular weight bands (Figure 6B). With any amount tested, the intensity of the M_r 70 000 band remained at ≥95% of the total staining. When 187 ng of thyromedin was analyzed (Figure 6B), the doublet pattern was found again. Because 1.0 ng of protein can be detected by the silver reagent (data not shown), the staining of 187 ng of thyromedin implied a high degree of homogeneity.

RP-FPLC Analysis of Thyromedin. Thyromedin from the phenyl-Sepharose step was analyzed for homogeneity by RP-FPLC on a ProRPC C_1C_8 column equilibrated with 0.1% TFA

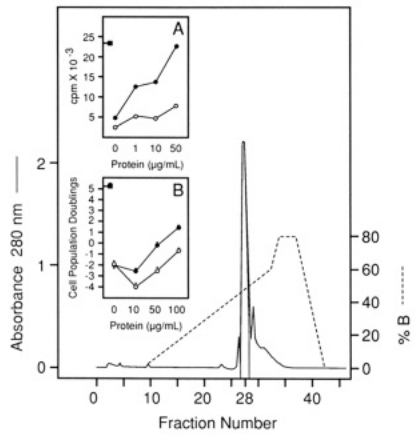

FIGURE 7: ProRPC RP-FPLC analysis of thyromedin from phenyl-Sepharose. A sample (2.0 mg) was applied to the column equilibrated with 0.1% (v/v) TFA and eluted with a linear gradient of solvent B (90% acetonitrile in 0.1% TFA) at a flow rate of 0.5 mL/min over 90 min. Fractions (1.25 mL) were collected and taken to dryness in a Savant vacuum concentrator. The major protein fraction (shaded area) was dissolved in acetic acid for amino acid sequencing. When bioactivity was required, a procedure described in the text was used. The insert A shows the bioactivity using the [^{3}H]TdR incorporation method (cpm) in PCM-0 (open circles) and PCM-10 (closed circles). Insert B shows activity by the cell number assay in PCM-0 (open circles) and PCM-10 (closed circles). The closed square in both assays shows PCM-0 with 5% horse serum.

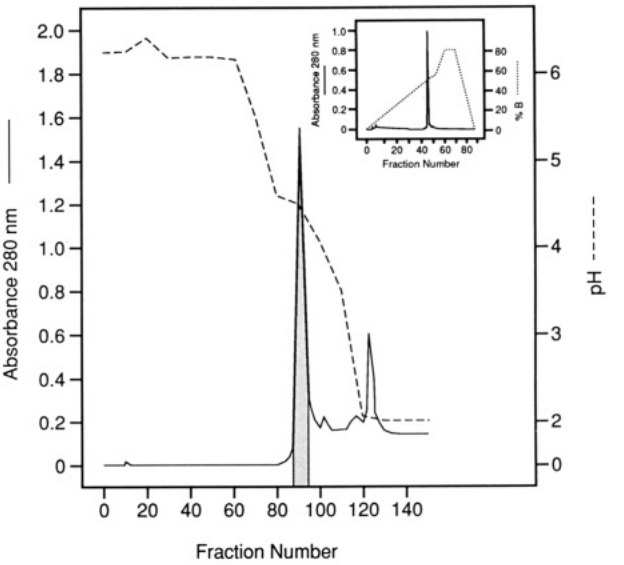

FIGURE 8: Chromatofocusing analysis of the thyromedin preparation from phenyl-Sepharose. The conditions of the chromatography are described in the text. The insert shows the application of 350 μg of p*I* 4.5 material (shaded area) to a Vydac C_4 column equilibrated with 0.1% TFA followed by washing for 30 min with this solution and elution with a linear gradient (90 min) of solvent B (90% acetonitrile in 0.1% TFA) at a flow rate of 0.5 mL/min. Fractions (0.6 mL) were collected and taken to dryness with a Savant vacuum concentrator. Fraction 46 was dissolved in acetic acid and used for amino acid sequencing.

(v/v) and eluted with a linear gradient of acetonitrile in 0.1% TFA (Figure 7). The elution profile showed a major A_{280nm} peak and two less pronounced peaks appearing immediately before and after. Aliquots of the major peak (fraction 28, shaded area) were analyzed by SDS–PAGE using both Coomassie Blue and silver staining methods (data not shown). The maximum concentration of protein applied per lane was 1.4 μg. With both staining methods, a major band of M_r 70 000 was identified. Also, RP-FPLC apparently separated the components of the doublet, allowing identification of only the single higher molecular weight protein when 175 ng was analyzed.

To test the major RP-FPLC peak for activity, a duplicate experiment to that shown in Figure 7 was done. Fractions were collected into 0.10 M sodium phosphate (pH 9.0) to neutralize the TFA and individual samples dialyzed immediately into PBS at 4 °C. When the [^{3}H]TdR incorporation assay was used, little growth was found in PCM-0 at thyromedin concentrations of 50 μg/mL (Figure 7A). In PCM-10, growth was dose-dependent. Also, activity was measured by the cell number method with similar results (Figure 7B). Although the pH 2.2/organic solvent conditions of RP-FPLC caused 80–90% inactivation of thyromedin, sufficient activity was present to confirm that the major RP-FPLC fraction was thyromedin.

Estimation of Isoelectric Point. Chromatofocusing was used to evaluate homogeneity and estimate p*I* (Figure 8). Thyromedin (2.0 mg) from phenyl-Sepharose was dialyzed against 1 L of 0.025 M histidine (pH 6.0) and applied at 0.5 mL/min to a 1.0 cm × 30 cm polybuffer PBE 94 exchanger column equilibrated with the histidine buffer. The column was washed and a gradient applied containing a 1:20 dilution of polybuffer 74 adjusted to pH 1.98. Fractions were collected (2.0 mL), and every tenth was used to determine pH. A major A_{280nm} peak eluted at pH 4.5 (shaded area, Figure 8) while another came at the end of the gradient (pH 2.0).

Assays of each peak for thyromedin activity were unsuccessful. Controls of polybuffer 74 solution alone at 1 → 10 000 dilutions without thyromedin supported growth of GH_1 cells in PCM-10 at levels equal to serum stimulation (data not shown). Attempts to reduce the polybuffer below concentrations causing interference were unsuccessful.

Each peak was concentrated by lyophilization and analyzed by SDS–PAGE. The p*I* 4.5 peak showed only the M_r 70 000 band (data not shown). The p*I* 2.0 peak gave a yellow oil which did not dissolve in aqueous solutions but did in 1% (w/v) SDS. SDS–PAGE of this material gave no Coomassie Blue staining (data not shown).

Amino Acid Sequencing. Thyromedin from concanavalin A–Sepharose contained a single component representing ≥95% of the sample (Figure 4). N^{α}-Amino acid sequence analysis of this preparation gave a major sequence as well as traces of others consistent with the presence of less abundant proteins. Seven amino acid residues of the major component were determined from 13 cycles of Edman degradation. They were N^{α}-Glu-Gln-(Thr)-Val-(X)-Trp-(X)-(X)-Val-(X)-Asn-(X)-Glu-COOH which was homologous to the partial sequence N^{α}-Glu-Gln-Thr-Val-Arg-Trp-Cys-Thr-Val-Ser-COOH determined for horse transferrin variant R (Chung & McKenzie, 1985). The ProRPC RP-FPLC purified fraction (shaded area, Figure 7) also was analyzed. The sequence determined was N^{α}-Glu-Gln-Thr-Val-(X)-Trp-(X)-Thr-Val-(X)-(X)-(X)-Glu-COOH. A portion of the lyophilized p*I* 4.5 peak from chromatofocusing (shaded area, Figure 8) was applied to a Vydac C_4 RP-HPLC column equilibrated with 0.1% TFA. The column was eluted with a linear gradient of acetonitrile (insert, Figure 8). A single A_{280nm} peak was collected into fraction 46 and used for sequencing. Sixteen cycles identified the sequence N^{α}-Glu-Gln-Thr-Val-Arg-Trp-Cys-Thr-Val-Ser-Asn-His-Glu-Val-Ser-Lys-COOH.

Amino Acid Composition. Amino acid composition of two RP-FPLC-purified thyromedin preparations were compared to that reported for equine transferrin variant R (Chung & McKenzie, 1985). Cysteine and methionine were underestimated because performic acid oxidations were not done. Comparisons of two thyromedin preparations to the amino acid

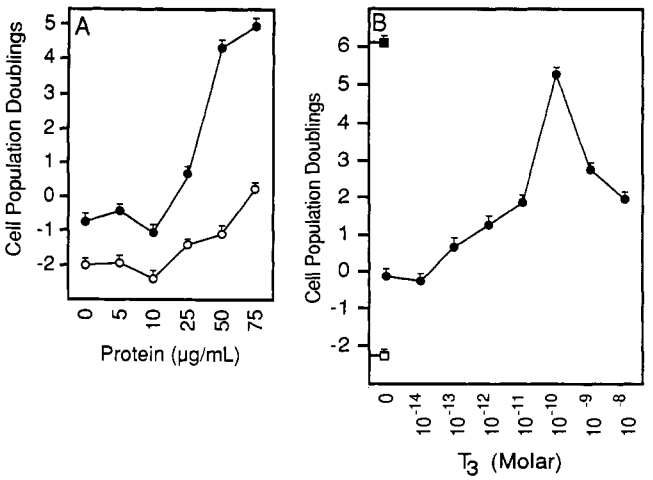

FIGURE 9: Dose–response growth of the GH_1 cells in thyromedin-containing serum-free medium. (A) Growth in PCM-0 (open circles) and PCM-10 (closed circles) in response to increasing concentrations of thyromedin from the phenyl-Sepharose step. (B) Growth in PCM-0 containing 50 μg/mL thyromedin with increasing concentrations of T_3. The closed square shows control growth in PCM-0 containig 5% horse serum; the open square is in PCM-0 only. In both experiments, the data were averages of triplicate plates (±SD, bars). The assay in (A) was 6 days; (B) was 7 days.

composition of horse serum transferrin variant R indicated marked similarities. The amino acid compositions of equine transferrin R and of two separate thyromedin preparations are listed, respectively. They were the following: Asx (70, 75, 75); Thr (31, 34, 36); Ser (46, 44, 46); Glx (65, 72, 64); Pro (32, 38, 41); Gly (50, 49, 53); $^1/_2$-Cys (37, 14, 14); Val (46, 48, 44); Met (7, 4, 4); Ile (21, 19, 20); Leu (56, 60, 58); Tyr (25, 29, 31); Phe (27, 24, 31); His (20, 20, 23); Lys (53, 54, 53); Arg (24, 34, 31).

Dose–Response Effects of Thyromedin and T_3 in PCM-0 and PCM-10. The dose–response effects of purified thyromedin in PCM-0 and PCM-10 were evaluated with GH_1 cells. In PCM-0, addition of 75 μg/mL increased cell survival to the initital seed density (Figure 9A). The same concentrations added to PCM-10 resulted 4.5 CPD/6 days which was 85% of the growth rate seen in 5% (v/v) horse serum (Figure 9B); as little as 25 μg/mL caused a significant increase in cell growth in PCM-10. Thyromedin ED_{50} calculated from these data was 40 μg/mL or 0.62 μM.

When T_3 concentration effects were studied in PCM-0 containing 50 μg/mL thyromedin, 0.1 nM T_3 was optimum (Figure 9B). Below 0.1 nM T_3, growth was significantly reduced; above 0.1 nM T_3, the hormone inhibited.

Comparison of the Effects of Thyromedin and Commercially Prepared Transferrins. PCM-0 and PCM-10 both contain 10 μg/mL human transferrin which did not sustain GH_1 cell survival or growth. Increasing this level to 200 μg/mL was without benefit. Becauase transferrins of different species were not completely interchangeable (Ward & Kaplan, 1987), commercially available horse transferrin was assayed for activity.

Horse transferrin was recharged with ferric ions (Ward & Kaplan, 1987) before assay. Assay of up to 200 μg/mL iron-saturated horse transferrin showed no increased survival in PCM-0 compared to human transferrin. Further, 200 μg/mL horse transferrin did not support GH_1 cell growth in PCM-10.

Effects of Alterations in Iron Content on Thyromedin Specific Activity. The final preparation of equine thyromedin was not the expected salmon pink color of ferric ion saturated transferrin. Further, thyromedin showed little, if any, absorption at 465 nm, indicating a lack of iron (data not shown). Atomic absorption spectroscopy measurements of thyromedin preparations gave iron contents equivalent to 10–20% saturation assuming two Fe^{3+} per molecule.

In a series of assays, the specific activity of thyromedin was measured versus the iron content of protein and versus the iron content of the defined medium. The dose–response assay in standard "iron salts containing" PCM-10 showed a typical ED_{50} of 17 μg/mL (Figure 10A). Atomic absorption spectroscopy measurements determined the total iron present in PCM-10 was 64–65 ng/mL. Under these conditions, growth in PCM-10 exceeded that in PCM-0 by up to 4.5 CPD/7 days. When thyromedin was citrate-treated to reduce the total iron content to <2.5% saturation (Roop & Putnam, 1967) and assayed in "iron salts reduced" PCM-10 (total iron content <10 ng/mL), the ED_{50} concentration decreased to 150 ng/mL, and the differences between growth in PCM-10 and PCM-0 were up to 4.0 CPD/7 days (Figure 10C). Conversely, when thyromedin was completely iron-saturated by the method of Ward and Kaplan (1987), and assayed in standard "iron salts containing" PCM-10, all biological activity was lost even at concentrations of 50 μg/mL (Figure 10C). Clearly, iron-saturated thyromedin did not support cell survival at seed density levels.

Discussion

Previously, this laboratory reported that pituitary cell mitogens were found in neutral pH extracts of uterus, kidney, hypothalamus, normal whole pituitaries, and hormone-responsive pituitary tumors (Leland et al., 1981; Sirbasku, 1978; Sirbasku & Leland, 1982; Sirbasku & Moo, 1982; Sirbasku et al., 1981, 1982) and in acetic acid extracts of some of these same tissues (Danielpour et al., 1984; Ikeda et al., 1984). Initially, these were proposed as possible mediators (estromedins) of the effects of steroid hormones on pituitary tumor cell growth (Sirbasku 1978; Sirbasku et al., 1982). With the increased interest in autocrine/paracrine growth factors and cancer cell proliferation [see review by Goustin et al. (1986)], we examined known mitogens for effects on pituitary tumor cells. This was especially important because Hinkle and Kinsella (1986) and Miller et al. (1987) had reported the thyroid hormone induction of autocrine factors which replaced the mitogenic effects of T_3. Also, GH_3 cells were known to produce IGF-I in culture, possibly as an autocrine factor (Fagin et al., 1987).

In this report, and in others (Riss et al., 1986, 1989), we addressed growth factor effects on T_3-stimulated pituitary tumor cell growth. As work progressed, it became apparent that these cells were refractory to the stimulatory effects of the well-known growth factors (Riss et al., 1986, 1989). From data in this report, it was evident that growth factors from five functional families of nonlymphoid mitogens did not substitute for either T_3 or thyromedin. In fact, mitogens such as EGF and bFGF were potent inhibitors in defined medium (Riss et al., 1989). These data, and others collected during the last 3 years, indicated that the mediators we sought were not classical polypeptide or small protein growth factors but instead were higher molecular weight proteins. Some of our earlier data had suggested this possibility (Sirbasku et al., 1981).

To identify mediators of hormone action, we returned to previous data showing that estrogen-dependent pituitary tumor growth occurred only in euthyroid animals (Sorrentino et al., 1976b). Suppression of thyroid hormones caused marked inhibition of estrogen trophic effects. Further, both estrogen and thyroid effects at physiological concentrations of hormone required the presence of some component(s) of serum (Amara

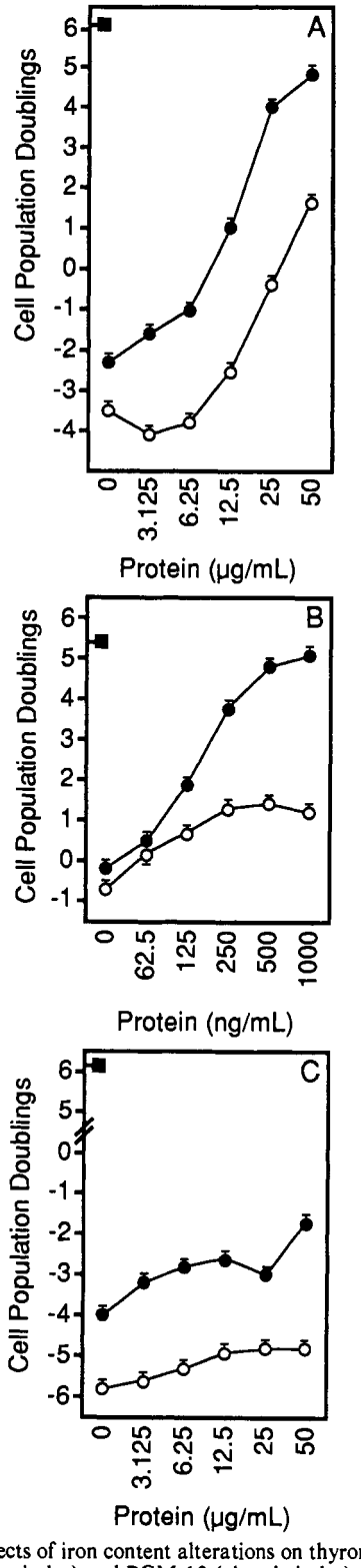

FIGURE 10: Effects of iron content alterations on thyromedin activity in PCM-0 (open circles) and PCM-10 (closed circles). (A) GH_1 cell growth in response to phenyl-Sepharose-purified thyromedin. The closed square shows growth in PCM-0 plus 5% horse serum. (B) Growth in response to citrate-dialyzed thyromedin. (C) Growth in response to citrate-dialyzed thyromedin which was iron-saturated with ferric ammonium citrate. In all experiments, the data are the averages of triplicate plates (±SD, bars). The assays were 7 days.

& Dannies, 1983; Riss & Sirbasku, 1989; Samuels et al., 1973). We set out to purify the hormone mediators directly from serum.

We began with the isolation of thyromedin because the effects of thyroid hormones were easily measured in serum-containing cultures (Kirkland et al., 1976; Riss & Sirbasku, 1989; Samuels et al., 1973). We developed an assay for thyromedin activity based on the assumption that the mediator acted in the presence of T_3 (i.e., PCM-10) but not in the absence (i.e., PCM-0). Application of this assay together with conventional chromatography methods led to the isolation of transferrin. The most impressive aspect of these findings was that thyroid hormone responsive growth was absolutely dependent upon addition of ≤75 μg/mL horse transferrin/thyromedin isolated in this study. This result was perplexing because human transferrin was a standard component present already in PCM-0 and PCM-10, and commercially prepared horse transferrin was inactive at 200 μg/mL. The most immediate question became the following: "How was the equine transferrin isolated in this study different than other transferrins?"

Comparison of our data to those of others characterizing transferrins suggested an interesting possibility. Thyromedin might be a variant form of horse transferrin. Equine serum transferrins are highly polymorphic. Initially, six homozygous phenotypes were identified by biochemical and immunologic methods (Baer, 1969; Baer & Schwendimann, 1972). They were designated Tf D/D, Tf F/F, Tf H/H, Tf M/M, Tf O/O, and Tf R/R. Later, the number of variants was extended to 10 including the D, F_1, F_2, F_3, G, H, J, M, O, and R forms of which the F phenotype was most common (Stratil & Glasnak, 1981).

Transferrins from other species also display polymorphism. The origins of these variations have been investigated. While it was known that the sialic acid/carbohydrate content differences accounted for the ovine variants cataloged (Spooner et al., 1975), the situation with the more numerous bovine variants was more complex (Maeda et al., 1980). Differences in bovine transferrin polypeptide lengths, or amino acid substitutions, were thought to be as important as sialic acid variations. With equine transferrins, the involvement of both sialic acids and amino acid substitutions has been described (Chung & McKenzie, 1985). Using electrophoretic, biochemical, and protein chemistry methods, these investigators have shown that the equine R and D forms differed in two internal amino acid substitutions (i.e., D:R; Asp:Gly and Glu:Gly).

A partial N^{α}-amino acid sequence was determined for the R variant, and from peptide map data, deduced to be the same as the D variant. Also, equine transferrin is known to have two heteropolysaccharide units per polypeptide (Hudson et al., 1973). Variations in the sialic acid content of these has special effects. Both the R and D variants demonstrated a doublet band pattern on polyacrylamide gel electrophoresis which was directly related to charge properties. The migration of both of these bands (designated "fast" and "slow") was altered by neuraminidase treatment. However, again two components were found only with new mobilities. Other investigators (Kaminski et al, 1981) studying equine transferrins have concluded that differences in electrophoretic mobilities may be due to more structural variations than sialic acid content or amino acid substitutions and deletions. Together, the data indicated that equine transferrins are complex variants differing in carbohydrate content and in subtle alterations in the polypeptide chains. At present, detailed purification and protein chemistry has only been reported with the R and D variants (Chung & McKenzie, 1985).

Irrespective of whether thyromedin represents one or more genetic variants of horse transferrin, an important question still remained. How did this form differ from the commercially

available material which was biologically inactive at any concentration assayed? Presumably, commercially prepared horse transferrin was prepared from large pools of serum obtained from many animals. This material should contain many, if not most, of the variants present in equine serum. We expected this to include the R and D variants.

One consideration became prominent. Although the transferrin isolated in this study showed a high degree of homogeneity, several lower molecular weight components were identified by SDS–PAGE (Figure 6). It was entirely possible that these were the biological activity responsible for the thyromedin effect. That this might be the case was especially troublesome because the concentration of thyromedin required for ED_{50} was in the 260–620 nM range. Even at 1% contaminant of a bioactive peptide or a small protein might be sufficient to cause growth. For this reason, we continued examination of the properties of horse serum thyromedin.

One type of analysis proved to be the key to the problem. Iron content determinations showed that thyromedin was only 10–20% saturated (i.e., at most, one in five molecules had two Fe^{3+} bound). We considered the possibility that thyromedin was biologically active in the *apotransferrin* state. Our first consideration was whether apotransferrin existed in our assay medium. PCM-10 prepared with standard "iron salts containing" F12–DME had 65 ng/mL total iron content. Assuming this was entirely Fe^{3+}, the amount was sufficient to saturate 46.8 μg/mL apotransferrin. Rapid and complete binding of Fe^{3+} to apotransferrin ($K_a = 10^{23}$) in tissue culture medium was expected because of the highly favorable conditions (Ward & Kaplan, 1987). Indeed, we found that 50–75 μg/mL thyromedin was required to saturate growth in "iron salts containing" medium. The existence of apotransferrin in our assays was possible.

We tested the putative role of apotransferrin by another method. If apotransferrin was the active agent, the specific activity of thyromedin should be enhanced by removal of the iron and assay in "iron salts reduced" medium. This was exactly what we found. Iron salts reduced PCM-10 contained <10 ng/mL total iron. The exact level remains unknown because of the limits of sensitivity of atomic absorption spectroscopy. Thyromedin was citrate-treated to reduce the iron content below the level of measurement by atomic absorption spectroscopy (i.e., equivalent to <2.5% saturation). Assay of citrate-treated thyromedin in "iron salts reduced" medium gave an ED_{50} of 150 ng/mL which was 2.3 nM (Figure 10B). This was a 110–270-fold increase in biological activity compared to iron-containing conditions. Further, 1.0 μg/mL iron-depleted thyromedin saturated the growth response in "iron salts reduced" medium rather than the usual 50–75 μg/mL when the metal was abundant in the assays.

In a final test, thyromedin was completely Fe^{3+}-saturated and assayed in "iron salts containing" medium. This resulted in the complete loss of biological activity (Figure 10C). If thyromedin was active in the apotransferrin state, this was the expected result. Further, these data provied an explanation of why commercially prepared horse and human transferrins were inactive. They were completely iron-saturated before addition to the assays. Also, these data tended to rule out the hypothesis that low-level contaminants were responsible for the activity in our thyromedin preparation. It was unlikely that the contaminant was activated more than 100-fold by removal of iron from the preparation and the assay and completely suppressed by recharge with ferric ammonium citrate and assay in standard iron salts containing defined medium.

The data presented have important biological consequences. First, in the more than 40 years of transferrin research, no specific physiological function for apotransferrin has been identified other than to act as a carrier for iron (Baker et al., 1987). Our data were evidence of a new function in regulation of hormone-dependent cell growth. Second, it is known that apotransferrin is formed by most, if not all, mammalian cells via internalization and recycling of the diferric transferrin/receptor complex (Ciechanover et al., 1983). If, as expected, apotransferrin is produced by this same process by pituitary cells, the possibility of thyroid hormone regulation of recycling becomes a prominent consideration.

Registry No. T_3, 6893-02-3; Fe, 7439-89-6.

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