complexes, which may be the reason for the strict conservation of Tyr70 in AATases (Kondo et al., 1987; Fotheringham et al., 1986).

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Thyroid Hormone Dependent Pituitary Tumor Cell Growth in Serum-Free Chemically Defined Culture. A New Regulatory Role for Apotransferrin†

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ABSTRACT: Thyroid hormone dependent GH_1 rat pituitary tumor cell growth in serum-free chemically defined medium required a serum-derived mediator (i.e., thyromedin) which was identified as transferrin [Sirbasku, D. A., Stewart, B. H., Pakala, R., Eby, J. E., Sato, H., & Roscoe, J. M. (1990) *Biochemistry 30*, 295-304]. The transferrin isolated was consistent with the equine R or D variants and was biologically active only as apotransferrin (apoTf). To determine if other variants of horse transferrin also were thyromedins, a purification was developed which yielded seven separate forms. Initially, only four of these had activity when assayed in standard "iron salts containing" medium (ED_{50} values of 290–1160 nM). To further assess activity, the iron contents of all seven were altered either by saturation with ferric ammonium citrate or by citrate/acid depletion of the metal ion. Thereafter, potencies were compared in "iron salts containing" and "iron salts reduced" media. All seven variants proved to be active as apoTf. Bioassays in which apoTf was maximized showed ED_{50} values of 2.1–3.8 nM. Conversely, assays in which thyromedins were converted to Tf·2Fe showed no activity. Previously, the only known physiological function of apoTf was that of a carrier/detoxifier of iron; this study indicates a new role in hormone-dependent pituitary cell growth.

Previous studies (Hayashi & Sato, 1976; Hayashi et al., 1978; Bottenstein et al., 1979; Hayashi, 1984) established that rat pituitary tumor cells grew in serum-free chemically defined media supplemented with several hormones including triiodothyronine (T_3),[1] Tf·2Fe, and insulin or insulin-like growth factors. More recently, we have studied T_3-dependent pituitary cell growth in serum-free medium and in cultures supplemented with hormone-depleted sera (Riss et al., 1986, 1989; Riss & Sirbasku, 1989). When we compared our results to those of others, problems were apparent.

Using the GH_4C_1 (Tashjian et al., 1970) and the GH_3 (Tashjian et al., 1968) cell lines, we found they survived

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[1] Abbreviations: apoTf, apotransferrin; CPD, cell population doublings; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; F12–DME, 1:1 (v/v) mixture of Ham's F12 nutrient medium and Dulbecco's modified Eagle's medium containing 2.2 g/L sodium bicarbonate and 15 mM HEPES, pH 7.2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; p*I*, isoelectric point; PTH, phenylthiohydantoin derivatives of amino acid residues; RP-HPLC, reverse-phase high-pressure liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tf·2Fe, diferric transferrin; T_3, triiodothyronine; TFA, trifluoroacetic acid.

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passage into serum-free culture only after a period of adaptation, required supraphysiological concentrations of T_3, and rapidly progressed to hormone-autonomy (Riss et al., 1989). Also, studies with the highly T_3-responsive GH_1 cells revealed still another problem. These cells failed to survive in any defined media, even those containing T_3 and saturating concentrations of insulin and Tf·2Fe (Riss et al., 1989; Stewart & Sirbasku, 1987, 1988a,b). These observations were in sharp contrast to the reports of T_3-responsive growth in serum-containing culture (Samuels et al., 1973; Hinkle & Kinsella, 1986; Riss & Sirbasku, 1989).

Because polypeptide growth factors are present in serum [see review by Gospodarowicz and Morgan (1976)], and are known to affect tumor cell growth [see review by Goustin et al. (1986)], several were assayed for mitogenic activity with rat pituitary tumor cells (Riss et al., 1986, 1989) and for the ability to substitute for T_3 in the defined media (Sirbasku et al., 1991b). In summary, growth factors did not stimulate proliferation nor did they substitute for the thyroid hormone requirement. Clearly, other factors in serum were responsible for supporting T_3-responsiveness.

These observations led to our first reports of "thyromedins" or mediators of the response to thyroid hormones (Stewart & Sirbasku, 1987, 1988a,b). In a recent report (Sirbasku et al., 1991b), a single form of thyromedin was purified from horse serum and was identified as transferrin. The data suggested that thyromedin/transferrin represented 1, or possibly 2, of the 11 known equine genetic variants (Baer, 1969; Baer & Schwendimann, 1972; Braend, 1964; Coddeville et al., 1989; Chung & McKenzie, 1985; Stratil & Glasnák, 1981; Stratil et al., 1984). Also, the thyromedin activity of the transferrin isolated was expressed only under iron-reduced conditions; iron saturation completely inactivated thyromedin. However, before concluding that thyromedin activity resided with only one or two apoTf variants, it was necessary to isolate a number of forms and test each for activity after alterations in iron content. We report the isolation of seven forms of equine serum transferrin and show that all supported thyroid hormone dependent cell growth as apoTf under iron-reduced medium conditions.

Experimental Procedures

Materials. Phenyl-Sepharose CL-4B, DEAE-Sepharose CL-4B, polybuffer 74, and polybuffer exchanger PBE 94 were purchased from Pharmacia. Anion-exchange AG 1-X 8 resin (100–200 mesh) was from Bio-Rad. Bovine insulin, selenous acid, and human Tf·2Fe were from Collaborative Research. Bovine serum albumin, soybean trypsin inhibitor, T_3, trizma base, and ethanolamine were from Sigma. Crude pancreatic trypsin was purchased from ICN. Trypsin–EDTA in Hank's balanced salts solution without calcium and magnesium salts was from Irvine Scientific and contained 0.5 g/L trypsin (1:250) and 0.2 g/L EDTA. Standard F12–DME containing 0.05 mg/L $Fe(NO_3)_3 \cdot 9H_2O$ and 0.417 mg/L $FeSO_4 \cdot 2H_2O$ was from GIBCO. Medium prepared with this powder was "iron salts containing" and had 65 ± 10 ng/mL total iron by atomic absorption spectroscopy (Galbraith Laboratories). The same powder without the iron salts (GIBCO) was used to prepare "iron salts reduced" medium (total iron ≤10 ng/mL). The Fe^{3+} salts added to defined media and used to saturate thyromedins and serum were from Sigma. Reagent-grade ammonium sulfate, sodium chloride, hydrochloric acid, and sodium bicarbonate were from Fisher. Dialysis membranes (Spectropor 3, molecular weight cutoff 3500) were from Spectrum Medical Industries. The filters used for sterilization were 0.22-μm pore diameter Millex-GV disks (Millipore).

Water and Stock Solutions. Water was purified sequentially by reverse osmosis, by a Barnstead/Thermolyne purification system, and by distillation in a Bellco glass apparatus with Teflon connectors. This reduced the total iron to ≤10 ng/mL.

The following solutions were prepared and stored at −20 °C in aliquots required for 200 mL of medium: bovine serum albumin, 60 mg/mL in water; ethanolamine, 10 mM in water; T_3, 10.0 μM in 0.01 N sodium hydroxide; bovine insulin, 10 mg/mL in water; selenous acid, 10 μg/mL in water; human Tf·2Fe, 10 mg/mL. To make defined medium, these were diluted into F12–DME and sterilized by membrane filtration immediately before use.

Stock Cell Cultures. GH_1 cells were from the American Type Culture Collection. Stocks were grown in standard "iron salts containing" F12–DME supplemented with 2.5% (v/v) fetal bovine and 12.5% (v/v) horse sera. Sera were from GIBCO.

The cells were grown at 37 °C in a humid atmosphere of 5% (v/v) CO_2 and 95% (v/v) air and passed every fourth day at 1.5×10^6/100-mm dish (Corning) containing 20–25 mL of medium. Subcultures were done by aspirating the medium, washing with 10 mL of saline, and adding 1.5 mL of trypsin–EDTA for 2–3 min at room temperature. The trypsin was stopped with 8.5 mL of F12–DME containing 15% (v/v) serum, cells were collected by centrifugation at 500*g* for 5 min, the supernatant was removed, and the pellet was dispersed in 10 mL of F12–DME. After cell number determination (Coulter Counter Model ZBI), fresh cultures were initiated. The GH_1 cells were assayed bimonthly for mycoplasma (MycoTect kit, Gibco) and were free of contamination.

Serum-Free Cell Growth Assays. Only 4-day cultures of GH_1 cells were used to initiate serum-free bioassays. Two defined media were used in our study (Riss et al., 1986, 1989; Riss & Sirbasku, 1989). The first (PCM-10) was F12–DME supplemented with 10 μg/mL bovine insulin, 10 μM ethanolamine, 10 ng/mL selenous acid, 500 μg/mL bovine serum albumin, 10 μg/mL human Tf·2Fe, and 0.10 nM T_3 while the second (PCM-0) contained all the same components except T_3. GH_1 cells were released from the dishes by the same trypsin–EDTA method described above except that the trypsin was stopped by 3.0 mL of 0.2% (w/v) soybean trypsin inhibitor in saline. Then, 5.5 mL of PCM-0 was added, and the cells were collected by centrifugation as above. The pellet was suspended in 10 mL of fresh PCM-0, and the cells were collected again. The final pellet was suspended in PCM-0 and used to inoculate $(2.0–2.5) \times 10^4$ cells/35-mm dish (Corning) in a final volume of 3.0 mL. The dishes were incubated at 37 °C as above for 7 days. The data expressed in the figures represent the means of triplicates ±SD. The volumes of thyromedins assayed did not exceed one-tenth of the total and were sterilized by membrane filtration.

To terminate the GH_1 assays, each dish was harvested by addition of 0.2 mL of 2% (w/v) crude pancreatic trypsin in Dulbecco's phosphate-buffered saline, pH 7.2, containing 0.152 M EDTA and incubation for 2–3 min at room temperature before addition of 0.4 mL of horse serum. When the dishes contained serum, the medium was replaced with F12–DME before trypsin/EDTA treatment. The contents of each dish were diluted to 10 mL with Isoton II (Coulter Electronics) and cell numbers determined.

Initial cell numbers were obtained from triplicate dishes harvested 1–3 h after seeding. The average of these, and triplicate dishes harvested after 7 days, was used to calculate CPD. Negative CPD values denoted cell death, zero CPD

Table I: Summary of the Purification of Multiple Forms of Equine Transferrin

sample	vol (mL)	protein concn (mg/mL)	total protein (mg)	ED_{50} (μg/mL)	act. (units)	% act. yield	x-fold purification
thyroid hormone depleted horse serum	980	47.0	46100	650	70900	100	1
50–65% ammonium sulfate precipitate	310	42.0	13000	125	104000	147	5.2
DEAE-Sepharose							
peak I	12	5.2	62.4	62.4	1040	1.47	10.8
peak II	18	10.4	187	187	2340	3.30	8.1
peak III	65	15.0	975	25	39000	55.5	26
phenyl-Sepharose							
peak Ia[a]	10	2.3	23	40	575	0.8	16.2
peak Ib[a]	20	4.6	92	>100	NA[d]	NA	NA
peak IIa[b]	24	3.2	76.8	25	3070	4.3	26.0
peak IIb[b]	16	1.1	17.6	80	220	0.3	0.3
peak IIc[b]	30	8.6	258	>100	NA	NA	NA
peak IIIa[c]	84	3.6	302	20	15100	21.3	32.5
peak IIIb[c]	50	6.7	335	>100	NA	NA	NA

[a] Four preparations of DEAE peak I were pooled (234 mg of protein) before phenyl-Sepharose. [b] Three preparations of DEAE peak II were pooled (525 mg of protein) before phenyl-Sepharose. [c] One preparation of DEAE peak III was used (924 mg of protein). [d] NA denotes not applicable, or could not be calculated.

indicated survival at inoculation densities, and positive CPD signified replication above seed densities. The ED_{50} of thyromedins with GH_1 cells was estimated as half the difference between the initial cell numbers and those after 7 days. One unit of thyromedin was required to achieve ED_{50}.

Thyroid Hormone Depleted Horse Serum. Horse serum was depleted of thyroid hormones as described (Samuels et al., 1979). Briefly, Bio-Rad AG-1 X 8 resin was washed with distilled water, autoclaved, and added (50 g/L) to sterile serum. The mixture was rotated in a Bellco roller bottle apparatus at 2 rpm for 70 h at 37 °C which depleted ≥80% of added ^{125}I-T_3. The serum was separated from the resin by filtration and stored at −20 °C.

Alterations in the Iron Content of Thyromedins and Horse Serum. To remove the iron from the thyromedin preparations, solutions (≤10 mL containing ≤10 mg/mL protein) were dialyzed twice for 48 h at 4 °C against 4 L of 0.10 M citric acid, pH 4.0 (Roop & Putnam, 1967), followed by dialysis for 48 h against two changes of 4 L of 0.05 M Tris-HCl, pH 7.4. When iron-saturated samples were required, samples (≤10 mg/mL) were brought to 0.01 M sodium bicarbonate and 0.10 mg/mL ferric ammonium sulfate or ferric ammonium citrate and incubated in the dark for 18 h at 25 °C (Ward & Kaplan, 1987). Excess iron was removed by dialysis at 4 °C against several changes of 4 L of 0.05 M Tris-HCl, pH 7.4.

To saturate horse serum with Fe^{3+}, the preparations were brought to 20 mM sodium bicarbonate and 0.25 mg/mL ferric ammonium citrate and incubated in the dark for 18 h at room temperature, and excess iron was removed by dialysis for several days against 0.05 M Tris-HCl, pH 7.4 at 4 °C.

SDS-PAGE. SDS-PAGE (Laemmli, 1970) was done with 0.75-mm-thickness 4% (w/v) stacking and 7–14% (w/v) gradient separating acrylamide gels at 200 V for 30–60 min at room temperature using a Bio-Rad Mini Protean II unit. Staining was done with 0.2% (w/v) Coomassie Brilliant Blue R-250 followed by destaining with 40% (v/v) methanol and 10% (v/v) acetic acid. The prestained proteins used to estimate molecular weights were myosin H chain (211 400), phosphorylase *b* (107 000), bovine serum albumin (69 300), ovalbumin (45 800), carbonic anhydrase (28 700), β-lactoglobulin (18 200), and lysozyme (15 400) from Bethesda Research Laboratories.

RP-HPLC. RP-HPLC was done at room temperature with a Rainin instrument. Solvent A was 0.1% (v/v) TFA in distilled water; solvent B was 0.1% TFA in 90% (v/v) HPLC-grade acetonitrile. Column eluants were taken to dryness with a Savant vacuum concentrator. The Vydac RP-HPLC column (4.6 mm × 250 mm, 5-μm particle size) was from Rainin. The μ-Bondapak CN RP-HPLC column (3.9 mm × 300 mm, 10-μm particle size) was obtained from Waters Associates. Acetonitrile HPLC-grade was obtained from Mallinckrodt; TFA was from Aldrich.

Protein Chemistry. Amino acid sequencing was done with an Applied Biosystems Model 477A instrument (Hunkapiller et al., 1983) with thyromedins which were reduced and cysteine-modified by S-pyridylethylations (Ingles, 1983; Andrews & Dixon, 1987).

Thyromedins were not chemically modified before amino acid composition analysis. Samples were brought to 0.1% TFA (v/v) and applied to the Vydac C_4 column and the A_{280nm} peaks dried and analyzed (Radhakrishna et al., 1989); tryptophan was not determined. Aliquots also were treated with performic acid before 6 N hydrochloric acid hydrolysis for estimation of cystine (Hirs, 1967). Equivalent aliquots were hydrolyzed at 110 °C in 6 N acid with 0.1% (w/v) phenol for 21 h. The analyses were done with an LKB Alpha Plus analyzer. Norleucine was included as an internal standard.

Protein Determination Method. Protein concentrations were estimated by the method of Bradford (1976) using a Bio-Rad kit and bovine serum albumin as standard.

Results

Purification of Horse Serum Thyromedins. Purification was done at 4–7 °C and is summarized in Table I.

Step 1. Thyroid hormone depleted serum showed an ED_{50} in PCM-10 of 650 μg/mL. At protein concentrations of ≤0.50 mg/mL, proliferation in PCM-10 exceeded that in PCM-0 by 2.6–3.7-fold (i.e., 1.4–1.9 CPD). At higher concentrations, the differences between PCM-10 and PCM-0 diminished, presumably because of residual thyroid hormones present even after anion-exchange resin extraction for 70 h.

Step 2. Solid ammonium sulfate (306.7 g) was added over 1 h with stirring to 980 mL of hormone-depleted serum (i.e., to 50% saturation). After 1 h of stirring, the precipitate was collected by centrifugation at 4500*g* for 45 min and discarded. The supernatant (970 mL) was treated with 98 g of ammonium sulfate over 1 h (i.e., to 65% saturation) and stirred for 1 h, and the precipitate was collected by centrifugation at 4500*g* for 1 h. It was dissolved in 150 mL of distilled water and dialyzed against 4 L of 0.10 M Tris-HCl, pH 8.6. The dialyzate was changed 4 times over 3 days. The red/brown

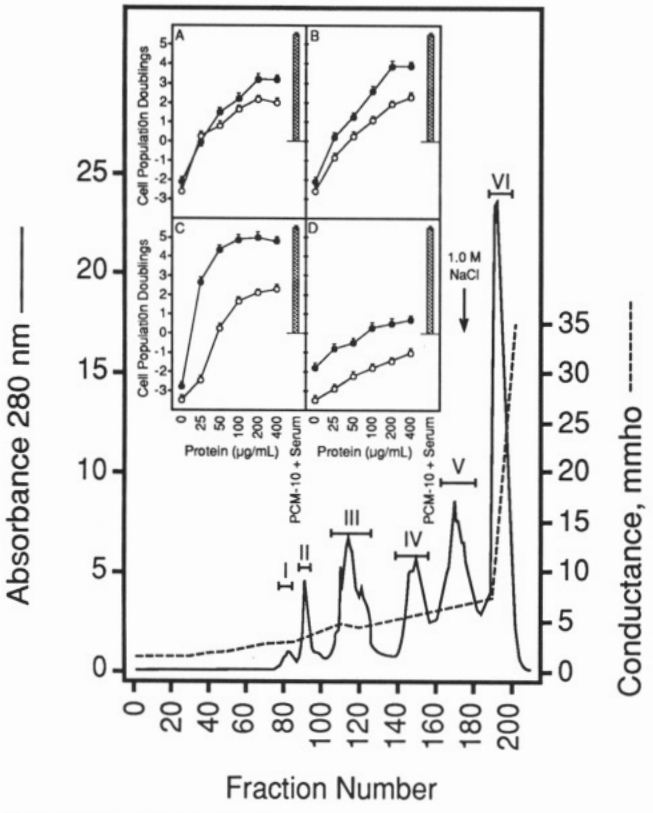

FIGURE 1: DEAE-Sepharose CL-4B of the 50–65% ammonium sulfate precipitate. The conditions are described in the text. Bars indicate pooled fractions. Inserts show GH_1 growth in PCM-10 (closed circles) and PCM-0 (open circles) containing protein from the pooled fractions: (A) peak I; (B) peak II; (C) peak III; (D) peak IV.

retentate (310 mL) was assayed for thyromedin activity. The ED_{50} was 125 μg/mL; at this concentration, cell numbers in PCM-10 exceeded PCM-0 by 13-fold (i.e., 3.7 CPD). Even at high concentrations, cell numbers in PCM-10 were 9 times greater than in PCM-0. Apparently, either ammonium sulfate precipitation or dialysis removed most of the residual thyroid hormones.

Step 3. A DEAE-Sepharose CL-4B column (5.0 cm × 51 cm, 1.0 L) was equilibrated with 0.10 M Tris-HCl, pH 8.6. The 50–65% ammonium sulfate precipitate was applied at a flow rate of 60–66 mL/h (25-mL fractions). The column was washed with 2 L of the Tris-HCl buffer and eluted with a linear gradient of 2.0 L of the equilibration buffer and the same volume of Tris-HCl containing 0.15 M sodium chloride.

Six major A_{280nm} peaks were identified (Figure 1). The first three were colored red/pink while the next were successively blue, grey, and then yellow. Selected fractions were analyzed by SDS–PAGE and Coomassie Blue staining (data not shown). Only an M_r 80 000 protein was detected in those corresponding to the maximum A_{280nm} of peaks I, II, and III. The fractions between peaks I and II contained the same protein while those between peaks II and III showed an additional M_r 69 000 band. As will be described below, this M_r 69 000 band was the major component found in thyromedin IIb. Beginning with peak IV, the staining indicated considerable heterogeneity.

The first five peaks were pooled as shown in Figure 1 and concentrated separately to 12–100 mL with an Amicon Ultrafilter using a YM-10 membrane and nitrogen gas pressure. These were used directly in the bioassays. Peak VI was dialyzed against 0.050 M Tris-HCl, pH 7.4, before concentration. Bioassays revealed significant activity in peaks I, II, and III (inserts A, B, and C, respectively, in Figure 1). In each case, proliferation above seed densities was measured, and growth in PCM-10 exceeded that in PCM-0. Peak III had the highest specific activity and showed the greatest effect in PCM-10 compared to PCM-0. The combined activity units in peaks I, II, and III were 60% of those in the original volume of horse serum. The total protein present (1224 mg) was 2.6% of serum.

Bioassay of peak IV showed another response pattern (insert D in Figure 1). Growth in PCM-10 still exceeded PCM-0, but proliferation surpassed inoculation densities by only 0.7 CPD; peaks V and VI showed the same pattern (data not shown).

Although SDS–PAGE suggested that DEAE-Sepharose peaks I through III contained predominantly one M_r 80 000 component, the homogeneity of each was further analyzed by chromatofocusing (pH 7.0–4.0). Figure 2A shows that peak I gave two components of p*I* 6.25 and p*I* 5.95 while peak II (Figure 2B) showed a major p*I* 5.83 component and second at p*I* 5.95. The p*I* 5.95 material may have originated from incomplete separation from DEAE peaks I and II (Figure 1). Finally, peak III was resolved into three components (Figure 2C). The major form was p*I* 5.25 with another at p*I* 5.40 and a minor component at p*I* 5.50.

Step 4. Hydrophobic interaction chromatography was used next to attempt further purification of DEAE-Sepharose peaks I, II, and III. A phenyl-Sepharose CL-4B column (2.5 cm × 90 cm, 442 mL) was equilibrated with 0.050 M Tris-HCl, pH 7.4, containing 3.0 M sodium chloride. Before the DEAE peaks were applied, each was dialyzed sequentially against 4 L of 0.050 M Tris-HCl, pH 7.4, containing 1.0 M sodium chloride for 48 h (one dialyzate change) and 4 L of the same buffer containing 3.0 M sodium chloride.

Four preparations of DEAE peak I were pooled for phenyl-Sepharose chromatography. The sample was applied, and after the column was washed with 475 mL of equilibration buffer, it was eluted with a gradient of 1450 mL of 0.050 M Tris-HCl, pH 7.4, containing 3.0 M sodium chloride and 1500 mL of the same buffer without the salt (flow rate of 45–66 mL/h, 25-mL fractions). Two well-separated peaks, Ia and Ib, eluted at conductances of 120 and 75 $m\Omega^{-1}$, respectively (Figure 3A); they contained 49% of the protein applied. The bioassay of peak Ia showed an ED_{50} of 40 μg/mL. At 50 μg/mL, Ia promoted 20-fold higher GH_1 cell numbers in PCM-10 than in PCM-0 (insert Ia in Figure 3A). In contrast, Ib at up to 50 μg/mL did not sustain cell survival in either PCM-0 or PCM-10 (insert Ib in Figure 3A).

Three preparations of DEAE peak II were pooled and applied to the same column. Three peaks eluted at conductances of 144, 104, and 90 $m\Omega^{-1}$ (IIa, IIb, and IIc, respectively, Figure 3B); they contained 67% of the protein applied. The bioassay of peak IIa gave an ED_{50} of 25 μg/mL and 4.2-fold higher cell numbers in PCM-10 than in PCM-0 (insert IIa in Figure 3B). Peak IIb showed a higher ED_{50} concentration, lower maximum effects in PCM-10, and less difference between PCM-10 and PCM-0 (insert IIb in Figure 3B). Peak IIc was nearly inactive, requiring 25–50 μg/mL to maintain seed density cell numbers in PCM-10 (insert IIc in Figure 3B).

DEAE peak III also was applied to the same column. Elution gave peaks IIIa and IIIb at conductances of 150 and 100 $m\Omega^{-1}$, respectively (Figure 3C); they contained 69% of the protein applied. With one-fourth of the preparations, another minor peak appeared immediately before IIIa (data not shown). Peak IIIa, which eluted at the highest conductance of any of the forms, was active with an ED_{50} of 20 μg/mL in PCM-10 (insert IIIa in Figure 3C). Peak IIIb did

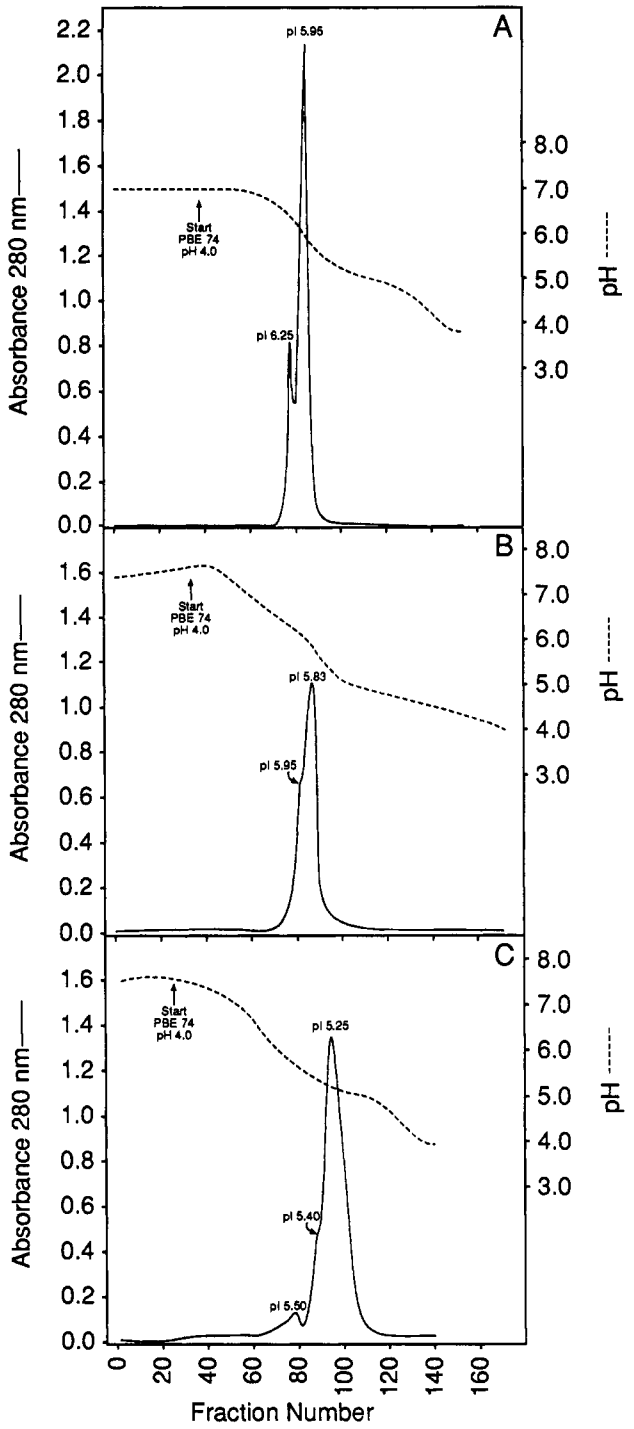

FIGURE 2: Chromatofocusing of DEAE-Sepharose peak I (A), peak II (B), and peak III (C). A PBE 94 exchanger column (1.4 cm × 25 cm, 38.5 mL) was equilibrated with 0.025 M imidazole, pH 7.4. Samples (47–61 mg) were dialyzed against this buffer for 24 h and applied at 1.1 mL/min (4.1-mL fractions). The column was washed with 100 mL of equilibration buffer and eluted with 560 mL of a 1 → 8 dilution of polybuffer 74, pH 4.0.

not achieve ED_{50} at 50 μg/mL in PCM-10 (insert IIIb in Figure 3C).

SDS–PAGE Evaluation of Homogeneity. All phenyl-Sepharose-derived samples were analyzed for homogeneity and molecular weight estimation by SDS–PAGE and Coomassie Blue staining (Figure 4). A major M_r 80 000 band was identified with all except IIb. This molecular weight was consistent with that of equine transferrin (Chung & McKenzie, 1985). The lower molecular weight components were estimated to be <5% of the total stain intensity. Peak IIb gave two stained bands of M_r 80 000 and 69 000. The higher molecular weight component was less intense and might represent IIa or IIc contamination arising from the incomplete separation of components by phenyl-Sepharose (Figure 3B). The M_r 69 000 band might be either a proteolytic product of the M_r 80 000 component or a different molecule.

Amino Acid Composition Analysis. Vydac C_4 RP-HPLC was used to prepare the phenyl-Sepharose-derived peaks for amino acid composition analysis (Figure 5A). In very case except IIb, a single A_{280nm} peak eluted at 195 ± 5 min containing approximately 50% of the applied material. All forms gave slightly asymmetric peaks which may have been due to variations in carbohydrate content (Coddeville et al., 1989). Nevertheless, SDS–PAGE of the major RP-HPLC A_{280nm} peak from each form confirmed a single Coomassie Blue stained band (insert in Figure 5A). Form IIb showed a number of A_{280nm} peaks (Figure 5B). The major of these, which eluted at 165 min, was analyzed by SDS–PAGE and shown to contain the M_r 69 000 substance. This material was used for amino acid composition analysis. The second most abundant peak, which eluted at 195 min, corresponded to the M_r 80 000 component identified in Figure 5A.

To summarize the composition data, horse transferrin variant R was compared to the average (±SD) of all phenyl-Sepharose-derived forms except IIb and finally to IIb alone. Residue values were, respectively: Asp (70, 67 ± 1, 64); Thr (31, 37 ± 1, 34); Ser (46, 42 ± 2, 47); Glu (65, 59 ± 1, 47); Pro (32, 31 ± 3, 44); Gly (50, 56 ± 2, 73); Ala (59, 65 ± 4, 58); Cys (37, 30 ± 10, 15); CTA (37, 33 ± 3, 22); Val (46, 43 ± 2, 33); Met (7, 5 ± 1, 10); Ile (21, 21 ± 1, 20); Leu (56, 53 ± 1, 56); Tyr (25, 30 ± 3, 25); Phe (27, 32 ± 1, 36); His (20, 23 ± 1, 28); Lys (53, 44 ± 1, 37); Arg (24, 38 ± 1, 43). There was close similarity between the average composition of the six forms (without IIb) and that of horse transferrin variant R (Chung & McKenzie, 1985). Only some residues of IIb differed from the average composition of the other six.

Partial N^{α}-Amino Acid Sequence Determination. Before being sequenced, the reduced and modified phenyl-Sepharose samples were applied to μ-Bondapak CN RP-HPLC. With the exception of IIb which gave three A_{280nm} peaks, the RP-HPLC profiles displayed a single component (data not shown). Partial N^{α}-amino acid sequencing gave the following results: peak Ia, Glu-Gln-Thr-Val-X-Trp-Cys-Thr; peak Ib, Glu-Gln-Thr-Val-Arg-Trp-Cys-Thr-Val-Ser; peak IIa, Glu-Gln-Thr-Val; peak IIc, Glu-Gln-Thr-Val-Arg-Trp-Cys-Thr-Val-Ser-Asn-His; peak IIIa, Glu-Gln-Thr-Val-X-Trp-Cys-Thr-Val-Ser; peak IIIb, Gln-Thr-Val-X-Trp-Cys-Thr-Val-Ser-Asn. All sequences were homologous to that reported by Chung and McKenzie (1985) for horse transferrin variant R and by Sirbasku et al. (1991b) for horse thyromedin prepared by our other method. With IIb, the first Edman degradation yielded PTH-Asn, PTH-Pro, and PTH-Leu; no unequivocal sequence was identified.

Iron Content of Thyromedins. Atomic absorption spectroscopy was used to measure the iron content of the phenyl-Sepharose-purified peaks (Table II). Percent saturations were calculated assuming all iron was Fe^{3+} and two Fe^{3+} per molecule, as expected for transferrin (Chasteen, 1983; Evans & Williams, 1978). Sufficient iron was found to account for 6.5–107% saturation. In parallel studies, citrate dialysis at pH 4.0 reduced the iron content to levels corresponding to <4.1% saturation (Table II). In experiments not presented, ferric ammonium citrate and ferric ammonium sulfate were used to Fe^{3+}-saturate the thyromedins; all acquired sufficient iron to account for ≥100% saturation.

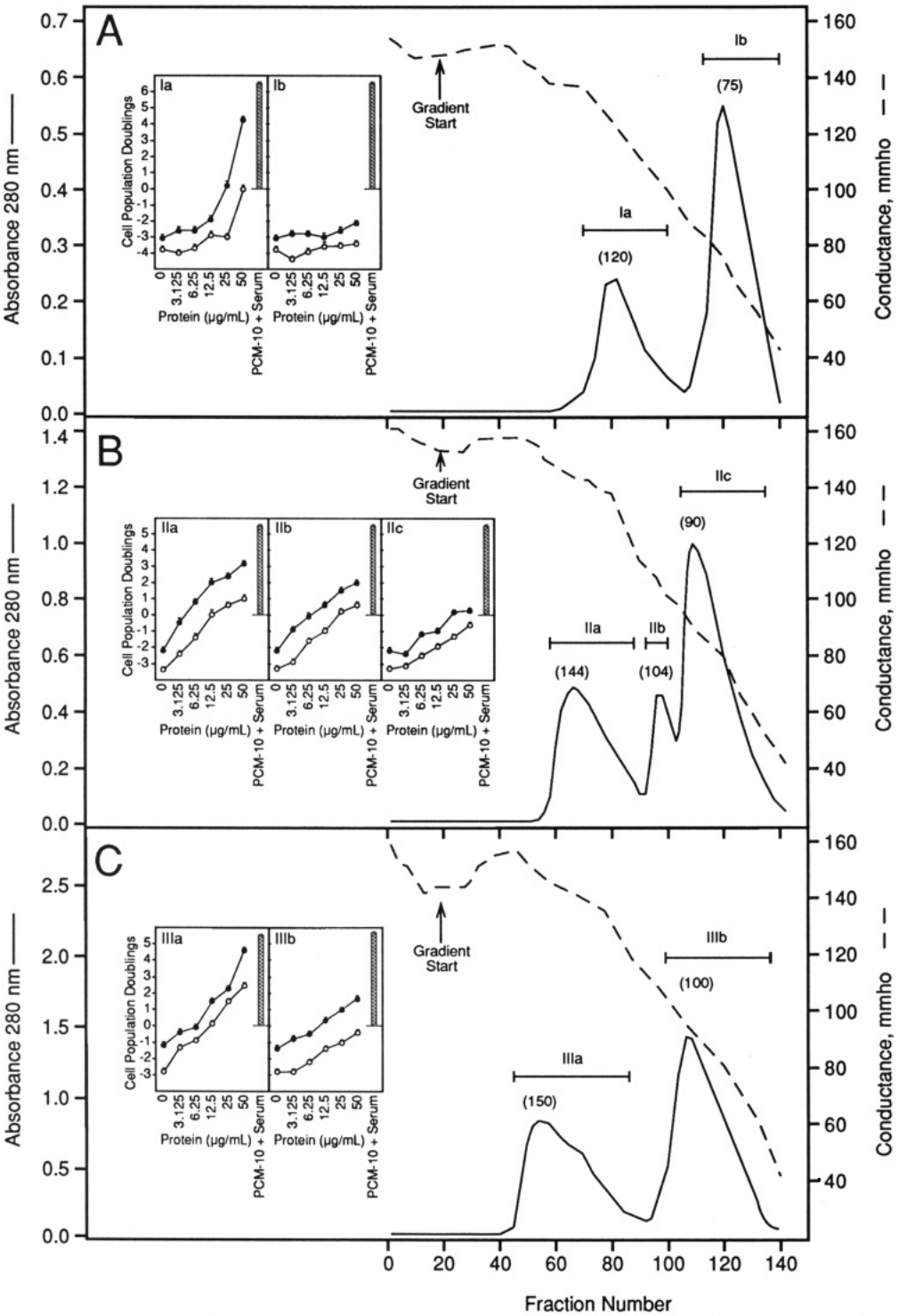

FIGURE 3: Phenyl-Sepharose CL-4B of DEAE peak I (A), peak II (B), and peak III (C). The conditions are described in the text. Values in parentheses show conductances of the maximum A_{280nm} of each peak. Bars show the fractions pooled. The inserts show the GH_1 cell growth in PCM-10 (closed circles) and PCM-0 (open circles) in response to phenyl-Sepharose peaks Ia and Ib (A), peaks IIa, IIb, and IIc (B) and peaks IIIa and IIIb (C).

Effects of pH on Thyromedin Activity. Aliquots of phenyl-Sepharose peak IIIb were dialyzed against buffers (2 × 4 L) ranging in pH from 2.7 to 11.8 for 48 h at 4 °C. Thereafter, the samples were returned to neutral pH by dialysis for 48 h against two changes of 4 L of 0.05 M Tris-HCl, pH 7.4, and assayed for activity. Dialyses at pH 2.7–6.0 utilized 0.10 M citrate buffers while those at pH 7.4 and 8.0 were done with 0.05 M Tris-HCl. Above pH 9.0, 0.10 M sodium bicarbonate/carbonate was used. Although IIIb was inactive before dialysis, acid treatment activated to an ED_{50} of 20 μg/mL. Furthermore, growth in PCM-10 exceeded that in PCM-0 by 800–1600% after dialysis at low pH. The potency of IIIb became equal to that of the most active forms (i.e., peaks Ia, IIa, and IIIa, Table I). Conversely, dialysis of IIIb at neutral or basic pH did not activate. The dose–response assays of these samples were equivalent to that of untreated IIIb shown in Figure 3C.

Phenyl-Sepharose Elution versus Iron Content. An aliquot of DEAE peak III (850 mg/50 mL) was dialyzed against 8 L of 0.1 M sodium citrate, pH 4.0, followed by dialysis against 4 L of 0.05 M Tris-HCl, pH 7.4, containing 1.0 M sodium chloride and against 4 L of the same Tris-HCl buffer con-

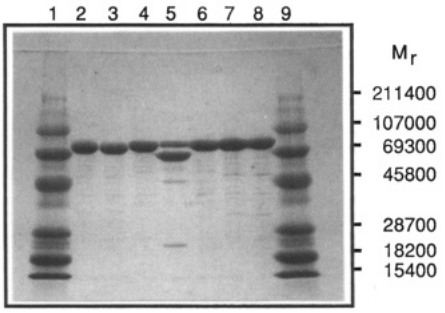

FIGURE 4: SDS–PAGE and Coomassie Blue staining of samples from phenyl-Sepharose. Lanes 1 and 9 received the mixture of molecular weight marker proteins. Lanes 2–8 received 1 μg each of peaks Ia, Ib, IIa, IIb, IIc, IIIa, and IIIb, respectively.

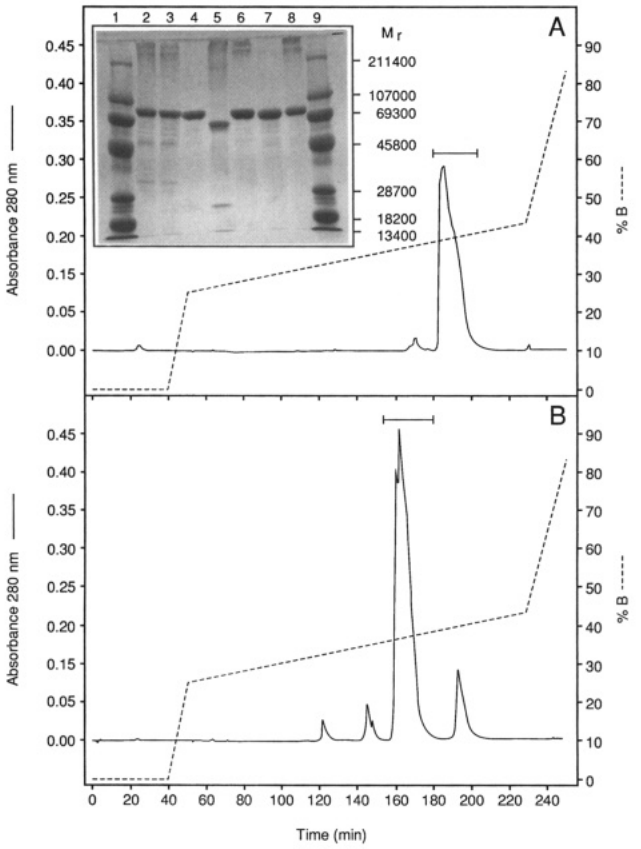

FIGURE 5: Vydac C_4 RP-HPLC of phenyl-Sepharose peak IIa (A) and peak IIb (B). The conditions are described in the text. The gradient concentration is shown as percent solvent B. The fractions pooled are shown by bars. The insert in (A) shows SDS–PAGE and Coomassie Blue staining of the major peaks from Vydac C_4 RP-HPLC with the seven thyromedins. Lanes 1 and 9 received the mixture of molecular weight marker proteins. Lanes 2–8 received 1 μg each of peaks Ia, Ib, IIa, IIb, IIc, IIIa, and IIIb, respectively.

taining 3.0 M sodium chloride. The sample was applied at a flow rate of 40 mL/h (25-mL fractions) to the phenyl-Sepharose column used above which was equilibrated with the high-salt buffer. After the column was washed with 450 mL of buffer, elution was done with a linear gradient of 1450 mL of the same buffer and 1500 mL of Tris-HCl without salt. The A_{280nm} revealed a profile not seen previously (Figure 6A). Although two major peaks still were identified, citrate treatment eliminated that corresponding to (inactive) IIIb. Growth assays of the peaks showed similar dose–response curves with ED_{50} values of 20–25 μg/mL in PCM-10 (inserts in Figure 6A). A total of 34 000 units of activity had been present in the sample before citrate treatment; twice that number was recovered. Since 77% of the protein was recovered from the column, the activity increase represented the activation of form IIIb by citrate treatment.

Table II: Summary of the Calculated Iron Saturation of the Equine Transferrins and Citrate-Treated Transferrins

sample	protein concn (mg/mL)	total iron (μg/mL)	calcd % saturation
phenyl-Sepharose-purified pools			
Ia	2.6	1.6	43
Ib	5.2	6.6	91
IIa	7.2	4.4	44
IIb	1.8	0.16	6.5
IIc	9.8	11.5	82
IIIa	6.3	4.7	53
IIIb	7.2	10.8	107
citrate-dialyzed phenyl-Sepharose-purified pools			
Ia	1.8	<0.10	<4.0
Ib	3.8	0.11	2.1
IIa	6.5	0.13	1.4
IIb	1.7	<0.10	<4.1
IIc	7.1	0.15	1.5
IIIa	5.8	<0.10	<1.2
IIIb	7.0	<0.10	<1.0

Conversely, iron saturation of DEAE peak III (496 mg) resulted in another pattern of elution from phenyl-Sepharose (Figure 6B). A single peak at conductance 110 $m\Omega^{-1}$ was observed which was the same elution position as (inactive) IIIb. This peak showed no activity in PCM-10 (insert in Figure 6B). A total of 19 800 units of activity had been present in the sample before iron treatment. Despite recovery of 82% of the protein, no activity was found, which indicated that form IIIa was rendered inactive by Fe^{3+} saturation.

Effect of Iron Content on Thyromedin Activity. Thyromedin IIIa was used to demonstrate the effects of changes in iron content on biological activity. Control assays of untreated IIIa in "iron salts containing" medium gave the expected ED_{50} of 20 μg/mL (Figure 7A) and PCM-10 growth which was 8-fold (i.e., 3.0 CPD) greater than in PCM-0. When this same sample was depleted of iron by citrate dialysis and assayed in "iron salts reduced" medium, the ED_{50} concentration decreased 117-fold to 170 ng/mL (Figure 7B), and growth in PCM-10 increased to 14-fold (i.e., 3.8 CPD) compared to PCM-0. Conversely, with Fe^{3+}-saturated thyromedin IIIa, assay in "iron salts containing" medium showed a different result (Figure 7C); IIIa failed to support cell survival at inoculation densities even in PCM-10. Finally, when citrate-dialyzed IIIa was assayed in standard "iron salts containing" PCM-10, the ED_{50} measured was 20 μg/mL (Figure 7D). When untreated IIIa was assayed in "iron salts reduced" medium, the ED_{50} decreased 100-fold to 200 ng/mL (Figure 7E).

The experiments in Figure 7A–D were repeated with the other six phenyl-Sepharose-purified thyromedins (Table III). Control assays with untreated thyromedins Ia, IIa, and IIb showed activity in "iron salts containing" PCM-10 (i.e., ED_{50} values from 17 to 80 μg/mL) while forms Ib, IIc, and IIIb were inactive. Whether the samples were active or inactive before citrate dialysis, assays afterward in "iron salts reduced" PCM-10 caused 100-fold increases in specific activities and increases in growth in PCM-10 versus PCM-0. When iron-depleted, all forms showed ED_{50} values of 180–325 ng/mL. Also, all citrate-treated samples were active in "iron salts containing" medium, albeit at 17–40 μg/mL. When these six citrate-dialyzed forms were iron-saturated and assayed in "iron salts containing" medium, activity was lost.

Effects of Exogenous Iron Salts on Thyromedin Activity.

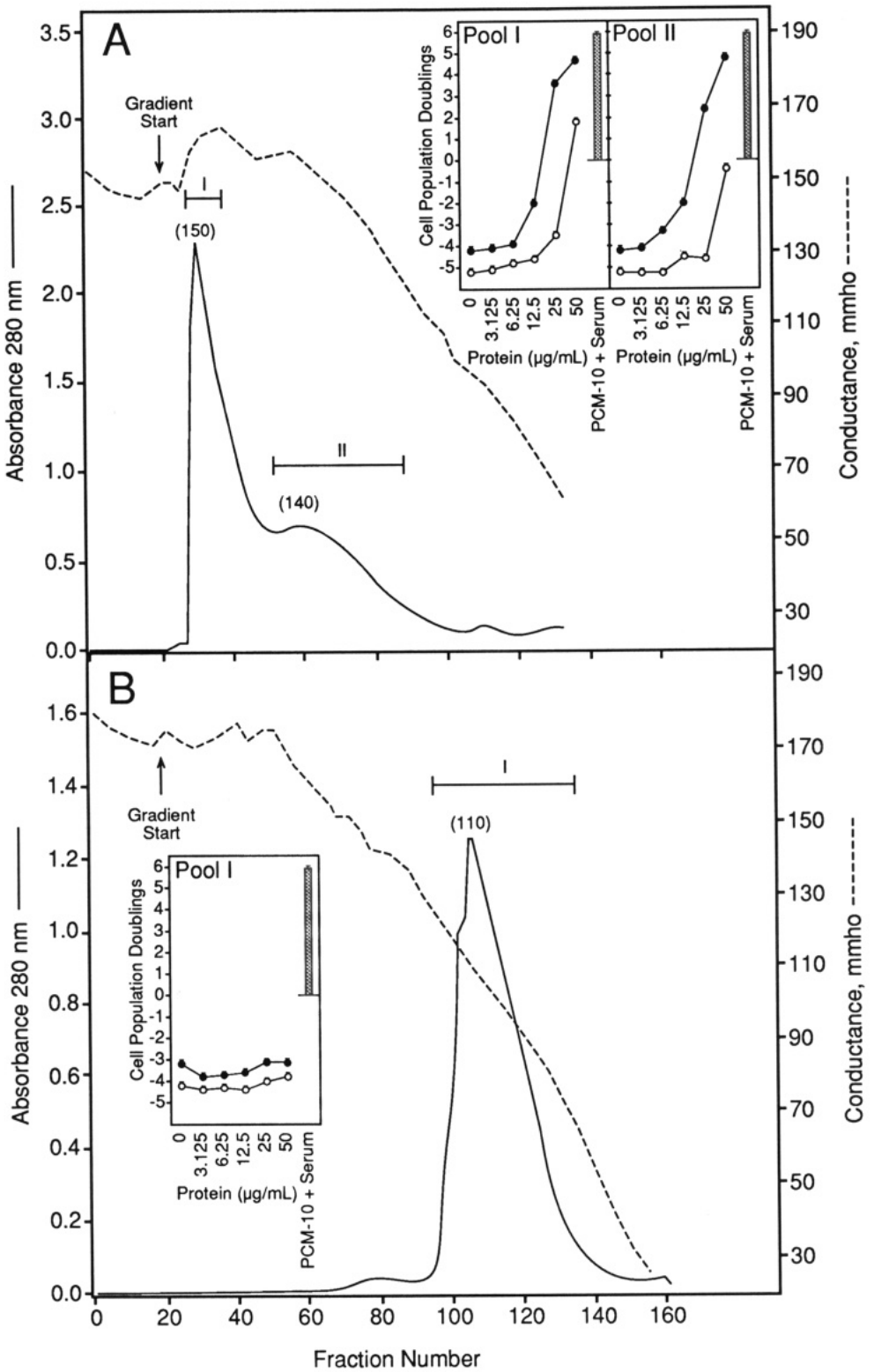

FIGURE 6: Phenyl-Sepharose of citrate-dialyzed (A) and Fe^{3+}-saturated (B) DEAE peak III. The conditions are described in the text. Bars show the fractions pooled for bioassay with GH_1 cells. The values in parentheses are the conductances of the fractions with maximum A_{280nm} for each pool. The inserts show GH_1 cell growth in PCM-10 (closed circles) and in PCM-0 (open circles) in response to (A) pools I and II from citrate-dialyzed DEAE III and (B) pool I from iron-saturated DEAE III.

In experiments not presented, the effects of Fe^{3+}/Fe^{2+} salts were investigated on citrate-dialyzed thyromedin IIIa activity in "iron salts reduced" PCM-10 and PCM-0. Form IIIa was studied at 12.5 and 25 μg/mL with the addition 2.5 or 5.0 μM ferrous sulfate, ferric chloride, ferric sulfate, ferric ammonium sulfate, or ferric ammonium citrate. All of these salts effectively inactivated IIIa. The dose–response effects of ferric ammonium citrate were studied with 1.0 μg/mL (0.125 nM) citrate-dialyzed IIIa; inhibition was half-maximum at 300 nM Fe^{3+}.

Effect of Deferoxamine on Thyromedin Activity. Deferoxamine mesylate (M_r 656) binds a single Fe^{3+} with much greater affinity than apoTf (Peter, 1985). However, Fe^{3+} associated with Tf·2Fe was not affected (Lipschitz et al., 1971; Perez-Insante & Mather, 1982). Assuming 10 ng/mL Fe^{3+} in "iron salts reduced" medium, addition of 0.2 μM deferox-

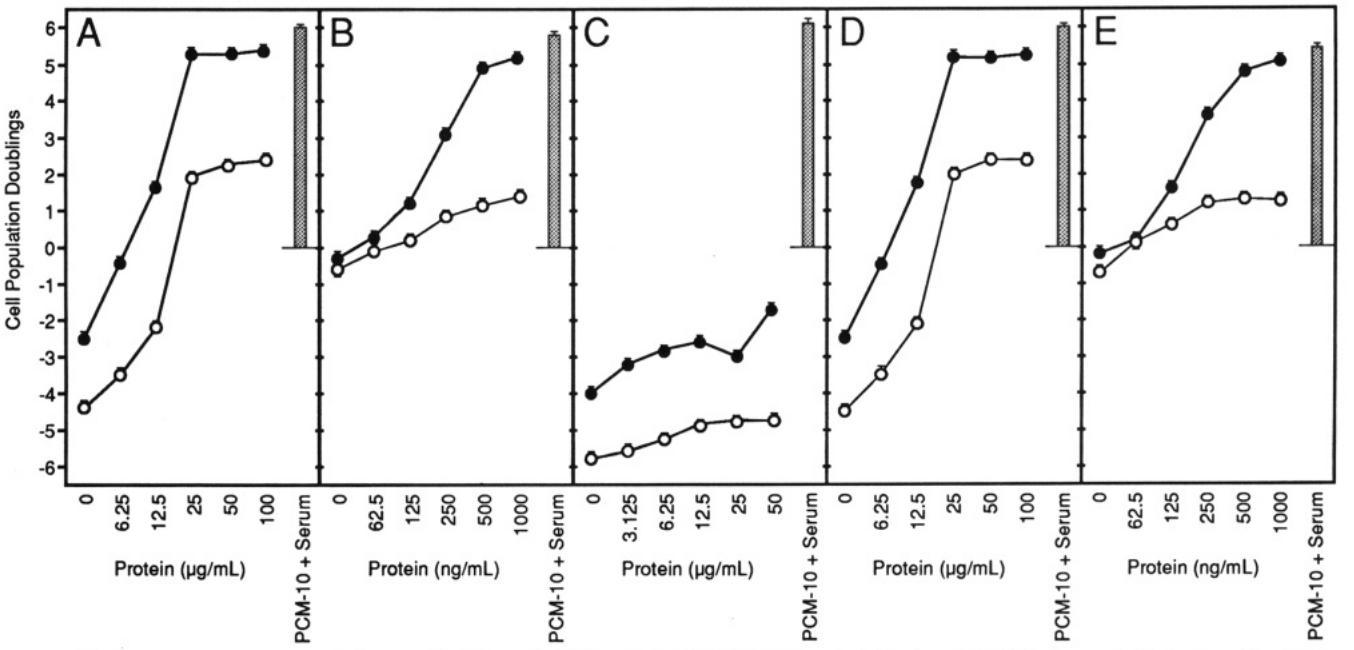

FIGURE 7: Dose–response assays of thyromedin IIIa with GH_1 cells in PCM-10 (closed circles) and PCM-0 (open circles) after alterations of the iron content of the protein and of the defined media: (A) untreated IIIa in "iron salts containing" media; (B) citrate dialyzed IIIa in "iron salts reduced" media; (C) citrate-dialyzed IIIa which was iron-saturated before assay in "iron salts containing" media; (D) citrate-treated untreated IIIa in "iron salts containing" media; (E) untreated IIIa in "iron salts reduced" media.

Table III: Thyromedin Activity versus Alterations in the Iron Content of the Protein and the Medium

thyromedin[a]	growth conditions[b]			
	A	B	C	D
Ia				
ED_{50}	40 μg/mL	180 ng/mL	>50 μg/mL	30 μg/mL
Δ[c]	3.2	3.4	0.1	2.9
Ib				
ED_{50}	>100 μg/mL	180 ng/mL	>50 μg/mL	40 μg/mL
Δ[c]	1.0[d]	3.4	0.2	3.3
IIa				
ED_{50}	25 μg/mL	325 ng/mL	>50 μg/mL	20 μg/mL
Δ[c]	2.0	3.5	0.3	3.3
IIb				
ED_{50}	80 μg/mL	300 ng/mL	>50 μg/mL	20 μg/mL
Δ[c]	1.0	3.7	0.1	3.2
IIc				
ED_{50}	>100 μg/mL	200 ng/mL	>50 μg/mL	20 μg/mL
Δ[c]	0.5	3.8	0.1	3.3
IIIb				
ED_{50}	>100 μg/mL	200 ng/mL	>50 μg/mL	17 μg/mL
Δ[c]	1.7	3.5	2.5[d]	2.8

[a] Thyromedin preparations were from phenyl-Sepharose. [b] The growth conditions were as follows: (A) untreated thyromedins assayed in "iron salts containing" medium; (B) citrate-dialyzed thyromedins assayed in "iron salts reduced" medium; (C) citrate-dialyzed thyromedins which were iron-saturated and assayed in "iron salts containing" medium; (D) citrate-dialyzed thyromedins assayed in "iron salts containing" medium. [c] Δ indicates CPD growth in PCM-10 minus that in PCM-0 measured at a saturating concentration of thyromedin. [d] Growth in PCM-10 did not equal inoculation density.

amine was expected to chelate this amount completely. In experiments not presented, preincubation of the medium with up to 64 μM deferoxamine had no effect either on the ED_{50} of citrate-treated IIIa or on the difference between growth in PCM-10 and PCM-0 in Tf·2Fe-containing media.

Activity of Commercially Prepared Equine Transferrin. In experiments not presented, equine transferrin (Sigma) was demonstrated to be inactive with GH_1 cells at concentrations of up to 200 μg/mL in standard "iron salts containing" PCM-10. However, upon citrate dialysis and assay in "iron salts reduced" PCM-10, an ED_{50} of 400 ng/mL was observed

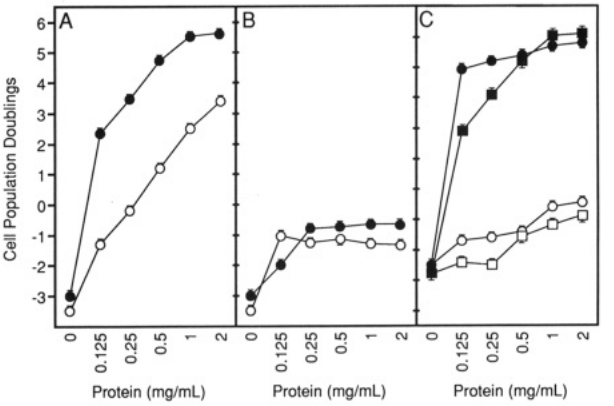

FIGURE 8: Growth of the GH_1 cells in defined media-supplemented horse serum: (A) assay in PCM-10 (closed circles) and in PCM-0 (open circles) containing thyroid hormone depleted serum; (B) the same experiment as (A) except the hormone-depleted serum was saturated with Fe^{3+} before the assay; (C) assay of iron-saturated horse serum protein (without hormone-depletion) in PCM-10 (open circles) and in PCM-0 (open squares) and in PCM-10 (closed circles) and in PCM-0 (closed squares) with control serum which was not iron-saturated.

in PCM-10. Transferrin is prepared commercially from Cohn's plasma (serum) fractions under acidic conditions in the presence of ethanol (Kistler & Friedli, 1980). These conditions may account for the lower specific activity of the commercial preparations. When citrate-dialyzed equine transferrin was assayed in standard "iron salts containing" medium, an ED_{50} of 35 μg/mL in PCM-10 was measured. When this preparation was iron-saturated and assayed in "iron salts containing" media, all activity was lost.

Effect of Iron Saturation on Thyroid Hormone Dependent Growth in Horse Serum. Growth promoted by thyroid hormone depleted serum was compared in "iron salts reduced" PCM-10 and PCM-0 (Figure 8A). At all protein concentrations assayed, growth in PCM-10 exceeded that in PCM-0 by 2.2–3.7 CPD. However, when the same serum was Fe^{3+}-saturated, it was unable to support thyroid hormone dependent GH_1 growth (Figure 8B). In another experiment,

GH_1 cell growth was measured in horse serum which had been Fe^{3+}-saturated without first depleting the thyroid hormones (Figure 8C). Under these conditions, serum-stimulated growth was completely inhibited even when T_3 was present.

Discussion

No physiological function for apoTf has been reported other than to serve as an acceptor/carrier for dietary and plasma iron (Baker et al., 1987; Chasteen, 1983; Morgan, 1981). Indeed, others have sought roles for apoTf in liver cell metabolism (Young & Aisen, 1981), in testicular cell growth (Perez-Insante & Mather, 1982; Mather & Sato, 1979), and in macrophage function (Nishisato & Aisen, 1982; Saito et al., 1986; Baynes et al., 1987). In a previous study (Sirbasku et al., 1991b), we purified a variant of apoTf/thyromedin from horse serum which was required for thyroid hormone dependent GH_1 cell growth in serum-free defined medium. In this report, we examined the possibility that many variants possess thyromedin activity by developing a new isolation protocol using pooled (commercial) serum which was genetically heterogeneous. This approach was in contrast to the isolation of specific transferrins described by others using serum from homozygous (Coddeville et al., 1989; Stratil et al., 1984) or heterozygous (Chung & McKenzie, 1985) horses.

Purification Protocol Changes Leading to Isolation of Seven Thyromedins. To accomplish the concurrent isolation of several variants, several changes were required in our previous methods (Sirbasku et al., 1991b). These were the following: (i) The GH_1 cells used in serum-free assays previously were harvested from serum grown stock cultures by trypsin–EDTA treatment, followed by addition of serum to stop the protease. Now, the trypsin was stopped with soybean trypsin inhibitor, and the cells were inoculated directly in serum-free medium; this increased sensitivity to thyromedins. (ii) The phosphate buffers used previously were replaced with Tris-HCl; yields increased at every step. (iii) Dialyses were extended to several days against large volumes which increased yields and chromatographic resolutions. (iv) Serum was depleted of thyroid hormones before beginning the isolation which doubled the initial units of thyromedin activity. (v) Affi-Gel Blue chromatography was eliminated; the loss of activity had been 95%. Also, the previous Sepharose 6B and concanavalin A–Sepharose steps became unnecessary due to improvements in the DEAE-Sepharose and phenyl-Sepharose methods. (vi) Our former method gave one final phenyl-Sepharose peak (20 mg of protein) which had <5% of the thyromedin activity of horse serum. In this report, seven thyromedins were obtained (>1100 mg of protein) which represented 25% of the initial activity.

Identification of Transferrin Variants. Although the transferrins isolated are presumed to be genetically separate, the designation "variants" remains tentative. We have not applied immunological methods, carbohydrate analyses, or peptide mapping to establish variant properties as done by others (Baer, 1969; Baer & Schwendimann, 1972; Coddeville et al., 1989; Chung & McKenzie, 1985; Stratil & Glasnák, 1981; Stratil et al., 1984).

Thyromedin Activity and ApoTf versus Tf·2Fe. Tf·2Fe is required for cell growth in serum-free culture (Barnes, 1987; Barnes & Sato, 1980a,b; Bottenstein et al., 1979). Because of these observations, it was essential to ask if our results are consistent with apoTf serving as a thyromedin rather than Tf·2Fe. The following data distinguish between these possibilities: (i) Several of the transferrins isolated by phenyl-Sepharose were not completely iron-saturated. If the iron-depleted proteins were thyromedin, the less saturated forms were expected to show activity, while the more saturated types would be inactive. Although this certainly was the case, the level of iron in the culture medium had important consequences. Activity was observed only when the amounts of apoTf added exceeded the available Fe^{3+} in "iron salts containing" medium. Since total iron concentration was approximately 65 ng/mL, the concentration of apoTf required was 40–50 μg/mL. Thyromedins Ia, IIa, IIb, and IIIa, which were ≤50% Fe^{3+}-saturated, showed ED_{50} values of 20–80 μg/mL and saturated growth at concentrations >50 μg/mL. In contrast, forms Ib, IIc, and IIIb, which were nearly iron-saturated, proved to be inactive in "iron salts containing" medium. (ii) Thyromedin IIIa contained nearly a 1:1 ratio of apoTf/Tf·2Fe. In "iron salts containing" medium, IIIa showed an ED_{50} of 20 μg/mL. If apoTf was active, assay in "iron salts reduced" PCM-10 would be expected to increase biological potency substantially because the Fe^{3+} in the medium was restricted. Indeed, this proved to be the case. Untreated thyromedin IIIa showed an ED_{50} of 180–200 ng/mL in iron-deficient medium. If Tf·2Fe had been the active thyromedin, a change in ED_{50} should not have been observed. (iii) The most direct test of the relative roles of apoTf and Tf·2Fe came from depleting the iron from the proteins and assaying in "iron salts reduced" medium. Although four thyromedins already were active in standard "iron salts containing" medium, under iron-restricted conditions we expected a loss of activity if Tf·2Fe was thyromedin, or a gain if apoTf was the mediator. The results were unequivocal. The four "active" forms became 100+ times more effective after Fe^{3+} depletion. The same studies with the "inactive" variants provided further confirmation. Whereas these did not support growth in PCM-10 even at 50 μg/mL when saturating iron was present, they became very active (ED_{50} of 180–325 ng/mL) under Fe^{3+}-depleted conditions. (iv) As additional evidence that apoTf was active rather than Tf·2Fe, the potencies of citrate-treated thyromedins were assayed again, but in "iron salts containing" medium. In every case, ED_{50} concentrations, which had been nanograms per milliliter in iron-deficient medium, increased more than 100-fold to the 17–40 μg/mL in iron-containing medium. If Tf·2Fe was the active thyromedin, the opposite results were expected. (v) Completely iron-deficient medium cannot be prepared by methods available currently. We were concerned that the residual metal might form sufficient Tf·2Fe to promote growth even in "iron salts reduced" medium. In fact, the concentration of available Fe^{3+} in iron-reduced medium is not known because of the limits of the sensitivity of atomic absorption spectroscopy and because elemental analysis does not distinguish between metallic, inorganic ion complexes and free ionic forms of iron. To further restrict the iron present in the medium, we added the potent chelator deferoxamine mesylate. When examined under iron-deficient conditions, citrate dialyzed IIIa showed the same ED_{50} as in medium without deferoxamine. (vi) As further evidence that apoTf was the active thyromedin rather than TF·2Fe or some minor contaminant [see discussion by Sirbasku et al. (1991b)], commercially prepared equine Tf·2Fe was assayed under a number of conditions. It was completely inactive when assayed in "iron salts containing" defined medium and activated >500-fold by citrate dialysis and assay in "iron salts reduced" medium. (vii) Although several lines of evidence demonstrated that Tf·2Fe was inactive, additional support for this conclusion was provided by our data. In PCM-10 containing 10 μg/mL human Tf·2Fe, enough transport protein was present to saturate the growth of other cell types in culture (Barnes & Sato, 1980a,b; Taub et al.,

1979; Ward & Kaplan, 1987). GH_1 cells not only failed to grow but was failed to survive. Also, when all seven citrate-dialyzed (active) thyromedins were brought to saturation with ferric ammonium citrate and assayed in "iron salts containing" defined medium, activity was lost invariably. Still other data indicated the same conclusion. The activity of citrate-dialyzed thyromedins was lost in "iron salts reduced" defined medium supplemented with increasing concentrations of ferric ammonium citrate. This same effect was demonstrated with commercially prepared apoTf (data not shown). (viii) All of the experiments showing no biological activity with Tf·2Fe were done in "iron salts containing" medium. When Tf·2Fe was assayed in "iron salts reduced" medium, significant growth was observed (Sirbasku et al., 1991a). Extracellular apoTf is formed by the well-characterized intracellular/endosome cycling of the Tf·2Fe/receptor complex (Ciechanover et al., 1983; May & Cuatracasas, 1985) which requires only minutes to complete and liberates extracellular apoTf. This same process most probably occurs with GH_1 cells (Sirbasku et al., 1991a). However, when Fe^{3+} is present in the medium, the released apoTf acquires iron and is thereby converted to inactive Tf·2Fe.

Effect of Iron Saturation on Thyroid Hormone Dependent GH_1 Cell Growth in Serum-Containing Culture. Rat pituitary cells have been grown in serum-containing cultures for many years and have retained thyroid hormone responsiveness (Hinkle & Kinsella, 1986; Miller et al., 1987; Riss & Sirbasku, 1989; Samuels et al., 1973; Sirbasku et al., 1991a,b). Clearly, some component(s) of serum was (were) responsible for maintenance of this property. Although our data supported the conclusion that apoTf was an important active agent, they did not exclude other regulators. We report here a convenient method for preparing apoTf reduced/depleted serum. Saturation of serum with Fe^{3+} converted apoTf to Tf·2Fe. When assayed, iron saturation completely inhibited serum-supported thyroid hormone dependent GH_1 cell growth with or without prior depletion of the hormones. These data support the conclusion that apoTf is thyromedin and indicate that this protein is the major regulator present in serum.

Relationship of the ApoTf/T_3 Observations to Other Mechanisms of Hormone-Dependent Pituitary Tumor Cell Growth. Other investigators have reported thyroid hormone induced "autocrine" factors which replaced completely the T_3 requirement of the GH_4C_1 and GC pituitary cells in culture (Hinkle & Kinsella, 1986; Miller et al., 1987). Also, we have reported an "autocrine" growth factor activity in the conditioned medium of the thyroid hormone responsive GH_3C_{14} cells and in extracts of the tumors (Danielpour et al., 1984). However, in these studies, either residual serum was present or several percent was in the medium. To better define the experimental conditions, we used serum-free methods (Riss et al., 1989) and were unable to identify constitutive or thyroid hormone inducible growth factors in the conditioned medium of GH_4C_1 cells. Further, growth factors from five major functional families of nonlymphoid mitogens either showed minimum growth effects or were inhibitory in serum-free medium (Riss et al., 1986, 1989; Sirbasku et al., 1991b). In view of the apoTf requirement in thyroid hormone dependent cell growth, we have suggested a new type of "autocrine factor" regulation in which an inactive serum protein is converted to an active form via intracellular processing (Sirbasku et al., 1991a).

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Initiation of Chondroitin Sulfate Biosynthesis: A Kinetic Analysis of UDP-D-Xylose:Core Protein β-D-Xylosyltransferase†

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ABSTRACT: The nature of the primary signals important for the addition of xylose to serines on the core protein of the cartilage chondroitin sulfate proteoglycan has been investigated. The importance of consensus sequence elements (Acidic-Acidic-Xxx-Ser-Gly-Xxx-Gly) in the natural acceptor was shown by the significant decrease in acceptor capability of peptide fragments derived by digestion of deglycosylated core protein with *Staphylococcus aureus* V8 protease, which cleaves within the consensus sequence, compared to the similar reactivity of trypsin-derived peptide fragments, in which consensus sequences remain intact. A comparison of the acceptor efficiencies (V_{max}/K_m) of synthetic peptides containing the proposed xylosylation consensus sequence and the natural acceptor (deglycosylated core protein) was then made by use of the in vitro xylosyltransferase assay. The two types of substrates were found to have nearly equivalent acceptor efficiencies and to be competitive inhibitors of each other's acceptor capability, with $K_m = K_i^{apparent}$. These results suggest that the artificial peptides containing the consensus sequence are analogues of individual substitution sites on the core protein and allowed the kinetic mechanism of the xylosyltransferase reaction to be investigated, with one of the artificial peptides as a model substrate. The most probable kinetic mechanism for the xylosyltransferase reaction was found to be an ordered single displacement with UDP-xylose as the leading substrate and the xylosylated peptide as the first product released. This represents the first reported formal kinetic mechanism for this glycosyltransferase and the only one reported for a nucleotide sugar:protein transferase.

Chondroitin sulfate proteoglycan (CSPG),[1] a major component of cartilage extracellular matrix, has a protein core of apparent molecular mass 340–370 kDa and is substituted with complex carbohydrates up to 10 times its protein mass (Upholt et al., 1979; Campbell & Schwartz, 1988). The linkage region of the characteristic chondroitin sulfate chain of CSPG consists of a serine-linked xylose–galactose–galactose–glucuronic acid tetrasaccharide followed by the repeating disaccharide, *N*-

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[1] Abbreviations: CSPG, chondroitin sulfate proteoglycan; HPLC, high-pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; PGHF, chondroitin sulfate proteoglycan deglycosylated with hydrogen fluoride; CS, chondroitin sulfate; CS,B, peptide B from chondroitin sulfate domain of core protein; CS,B-HF, CS,B deglycosylated with hydrogen fluoride; SDS, sodium dodecyl sulfate; GAG, glycosaminoglycan.

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