Lutter, L. C., Kurland, C. G., & Stöffler, G. (1975) *FEBS Lett. 54*, 144–150.
Massen, J. A., Schop, E. N., & Möller, W. (1981) *Biochemistry 20*, 1020–1025.
Moore, P. B., Lapel, M., Kjeldgaard, M., & Engelman, D. M. (1986) in *Structure, Function* and *Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 87–110, Springer-Verlag, Heidelberg and New York.
Nishikawa, K., & Ooi, T. (1980) *Int. J. Pept. Protein Res. 16*, 19–32.
Ohsawa, H., & Gualerzi, C. (1981) *J. Biol. Chem. 256*, 4905–4912.
Pohl, T., Brockmöller, J., & Kamp, R. M. (1986) in *Methods in Protein Sequence Analysis* (Walsh, K. A., Ed.) pp 609–614, Humana, Clifton, NJ.
Pon, C. L., Pawlick, R. T., & Gualerzi, C. (1982) *FEBS Lett. 137*, 163–166.
Rawlings, N., Ashman, K., & Wittmann-Liebold, B. (1983) *Int. J. Pept. Protein Res. 22*, 515–524.
Reimann, F., & Wittmann-Liebold, B. (1986) in *Advanced Methods in Protein Microsequence Analysis* (Wittmann-Liebold, B., Ed.) pp 118–125, Springer-Verlag, Heidelberg and New York.
Salnikow, J., Lehmann, A., & Wittmann-Liebold, B. (1981) *Anal. Biochem. 117*, 433–442.
Stöffler, G., & Stöffler-Meilicke, M. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 87–110, Springer-Verlag, Heidelberg and New York.
Wittmann-Liebold, B. (1986a) in *Practical Protein Chemistry—a Handbook* (Darbre, A., Ed.) pp 375–409, Wiley, New York.
Wittmann-Liebold, B. (1986b) in *Stucture, Function* and *Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 326–361, Springer-Verlag, Heidelberg and New York.
Wittmann-Liebold, B., & Kimura, M. (1984) in *Methods in Molecular Biology* (Walker, J. M., Ed.) Vol. 1, pp 221–242, Humana, Clifton, NJ.
Wittmann-Liebold, B., Ashman, K., & Dzionara, M. (1984) *Mol. Gen. Genet. 196*, 439–448.
Wittmann-Liebold, B., Hirano, H., & Kimura, M. (1986) in *Advanced Methods in Protein Microsequence Analysis* (Wittmann-Liebold, B., Ed.) pp 77–90, Springer-Verlag, Heidelberg and New York.
Wrede, P., & Erdmann, V. A. (1973) *FEBS Lett. 33*, 315–319.
Yaguchi, M., & Wittmann, H. G. (1978) *FEBS Lett. 71*, 251–255.
Yaguchi, M., Matheson, A. T., & Visentin, L. P. (1974) *FEBS Lett. 46*, 296–300.
Yonath, A., Saper, M. A., & Wittmann, H. G. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 112–127, Springer-Verlag, Heidelberg and New York.

Purification and Properties of an 18-Kilodalton, 1,25-Dihydroxyvitamin D_3 Modulated Protein from Embryonic Chick Intestine[†]

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ABSTRACT: An 18 000-dalton protein (pI = 5.1) shown previously to be modulated by 1,25-dihydroxyvitamin D_3 was purified to allow its further characterization. This protein from embryonic chick intestine was shown to comigrate during two-dimensional electrophoresis with an abundant protein from the intestine of 4-week-old chickens. The protein was purified from 4-week chick intestine and analyzed for amino acid composition, and 28 amino acids of its N-terminal sequence were determined. The N-terminal amino acid sequence had significant homology to cellular retinol binding protein II, an intestinal protein that has been recently sequenced. The purified 18-kilodalton protein was shown to bind retinol by fluorescence spectrophotometry. This 18-kilodalton protein is dramatically changed by 1,25-dihydroxyvitamin D_3 in the chick embryonic organ culture system. Therefore, further study of it may lead to a better understanding of vitamin A and D interaction and how 1,25-dihydroxyvitamin D_3 acts through proteins to stimulate intestinal calcium and phosphate transport.

The primary function of vitamin D is to regulate blood calcium and phosphate concentrations. The hormonally active form of the vitamin, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] plays its most important role in this process by stimulation of intestinal calcium and phosphate transport (DeLuca & Schnoes, 1984; DeLuca, 1986). We are interested in determining the molecular mechanism whereby 1,25-$(OH)_2D_3$ stimulates calcium and phosphate transport. To date, this mechanism is still unknown.

The chick embryonic duodenal organ culture (EDOC) is an ideal system in which to study the modulation of intestinal proteins by 1,25-$(OH)_2D_3$. In this system, it is possible to label

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; EDOC, embryonic duodenal organ culture; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; kDa, kilodalton; CaBP, vitamin D dependent calcium binding protein; pI, isoelectric point; M_r, molecular weight; CRBP II, cellular retinol binding protein II; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; DEAE, diethylaminoethyl; 2-ME, 2-mercaptoethanol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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radioactively proteins in the intestinal cells so that their metabolism may be monitored specifically and sensitively by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and autoradiography.

Protein synthesis and transcription inhibitors have both been shown to inhibit 1,25-$(OH)_2D_3$-dependent calcium uptake in the chick EDOC system (Corradino, 1973; Franceschi & DeLuca, 1981a), suggesting that mRNA and protein synthesis are required for 1,25-$(OH)_2D_3$ to stimulate calcium and phosphate transport. The vitamin D dependent calcium binding protein (CaBP) is the only protein known to be specifically induced by 1,25-$(OH)_2D_3$ in the intestine (DeLuca & Schnoes, 1984). Its induction has been shown to precede calcium uptake in the chick EDOC (Bishop et al., 1983). In this system after 20 h of exposure to 1,25-$(OH)_2D_3$, 0.55 nmol of $^{45}CaCl_2$ is taken up per milligram of tissue (Franceschi & DeLuca, 1981b). At least 0.138 nmol of CaBP/mg of tissue would be required to bind this much calcium. Bishop et al. (1984) showed that after 20 h of exposure to 1,25-$(OH)_2D_3$ the concentration of CaBP is only 0.128×10^{-3} nmol/mg of tissue. It is apparent that the amount of CaBP induced is not enough to account for the additional calcium taken up by the 1,25-$(OH)_2D_3$-treated duodena. Furthermore, there is other evidence that CaBP alone cannot account for the action of 1,25-$(OH)_2D_3$ on intestinal calcium transport (Harmeyer & DeLuca, 1969; Spencer et al., 1976).

Recently, an 18-kilodalton (kDa), 5.1 isoelectric point (p*I*) protein has been shown to be significantly changed by 1,25-$(OH)_2D_3$ in the chick EDOC system (Bishop et al., 1985). The 18-kDa protein was observed to change in two fundamentally different experiments. First, there is an 85% reduction in the radioactive protein if after treatment for 20 h with 1,25-$(OH)_2D_3$ the EDOC was labeled with [^{3}H]leucine for 10 min and then cultured with excess nonradioactive leucine for 4 h. Second, if the cultures are exposed to 1,25-$(OH)_2D_3$ for different lengths of time and labeled for 30 min with [^{14}C]leucine, the radioactive protein is reduced to 50% of the control value in 0.75 h. The concentration of radiolabeled protein increases and again falls back to 50% of the control value after 20 h of exposure to 1,25-$(OH)_2D_3$. Study of this 18-kDa protein may provide a lead into understanding 1,25-$(OH)_2D_3$-dependent calcium uptake.

In this paper, we prove that this embryonic, 1,25-$(OH)_2D_3$-modulated protein is identical with an abundant protein in 4-week-old chick intestine. We purified the protein from the chicken, obtained the N-terminal sequence, and have shown that this protein has N-terminal homology to the cellular retinol binding protein II found in rat small intestine (Li et al., 1986). We also found that the purified 18-kDa protein binds to retinol.

Materials and Methods

Chemicals. 1,25-$(OH)_2D_3$ was a gift from the Hoffmann-La Roche Co. (Nutley, NJ). *all-trans*-Retinol was purchased from Eastman Kodak (Rochester, NY). Chromatofocusing medium (i.e., PBE-94 ion-exchange resin and Polybuffer-74) and Sephadex G-50 superfine were obtained from Pharmacia (Uppsala, Sweden). Whatman DEAE-cellulose DE-52 was purchased from Fischer Scientific (Chicago, IL).

Animals. One-day-old white Leghorn chickens were obtained from Northern Hatcheries (Beaver Dam, WI) and maintained for 4 weeks on a 1.2% calcium/0.7% phosphorus vitamin D deficient diet consisting of sucrose (54.0%), soy isolate protein (22.0%), Solka Floc (7.0%), soybean oil (4.0%), $CaCO_3$ (2.4%), KH_2PO_4 (1.7%), $CaHPO_4 \cdot 2H_2O$ (1.0%), NaCl (iodized, 0.8%), L-cysteine (0.19%), L-tryptophan (0.08%), trace salts (0.7), and premix (5.5%) as previously reported (Hart et al., 1984). Four days before sacrifice, chickens were dosed intraperitonially with 2.5 μg of vitamin D_3 in 50 μL of propylene glycol per day. Animals were fasted for 12 h and killed by cervical dislocation.

Preparation of 4-Week Chick Intestinal Cytosol. Duodena were excised and rinsed with ice-cold 14 mM Tris-HCl, 50 mM NaCl, 5 mM KCl, and 1 mM 2-mercaptoethanol (2-ME), pH 7.4, and slit open, and the mucosa was scraped off. All subsequent protein purification steps were performed at 4 °C unless otherwise indicated. The mucosa was homogenized in 2 volumes of the above buffer and frozen. The mucosa was then thawed, rehomogenized, and centrifugated at 100000*g* for 2 h. Lipids were removed from the top, and the rest of the supernatant cytosol was frozen at −70 °C until use.

Preparation of ^{3}H-Labeled 19-Day Embryonic Chick Duodenal Cytosolic Proteins. The radiolabeled embryonic chick proteins were prepared as previously described (Bishop et al., 1985).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to assay qualitatively for the presence of the 18-kDa protein. This was done with 0.75-mm-thick mini gels cast and run in specialized equipment (Hoefer Scientific Instruments, San Francisco, CA). The gels consisted of a 5% stacking and an 18% separating gel and the buffer system of O'Farrell (1975).

Two-dimensional (2D) polyacrylamide gel electrophoresis was performed according to the method of O'Farrell (1975) by the Kendrick Laboratory (Madison, WI) as follows: For the first dimension, isoelectric focusing (IEF), using 1.5% pH 5–7, 1.5% pH 5–8, and 1.0% pH 3.5–10 ampholines from LKB Instruments (Baltimore, MD), was carried out at 400 V for 12 h followed by 800 V for 30 min. Forty nanograms of an internal standard, vitamin D dependent calcium binding protein (CaBP), M_r 27 000 and pI = 4.2 (Bredderman & Wasserman, 1964), was added to some of the samples. This standard is indicated by an arrow on the stained 2D gel (Figure 5B). The final tube gel pH gradient extended from pH 4.1 to pH 8.5 as measured by a surface pH electrode (Bio-Rad, Richmond, CA) on representative tubes.

For the second dimension, the following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards to the agarose which sealed the tube gels to the slab gels: myosin (200 000), phosphorylase A (94 000), catalase (60 000), actin (43 000), and lysozyme (14 000). These standards appear as fine horizontal lines on the stained 12% acrylamide slab gels. Gels were stained with Coomassie blue or treated with En^{3}Hance (Du Pont/New England Nuclear, Boston, MA) and dried.

Purification of the 18-kDa Protein from 4-Week-Old Chick Duodena. The chick duodenal cytosol solution was thawed and then heated to 60 °C with stirring and maintained at this temperature for 20 min. The solution was rapidly cooled to 4 °C and centrifuged at 20000*g* for 20 min. The supernatant fraction was then treated with 1.2 volumes of ice-cold acetone and stirred for 4 h. The resulting solution was centrifuged at 3000*g* for 30 min to collect the precipitate which was allowed to dry. The residue was then allowed to stir overnight in 10 mM Tris-HCl pH 7.5, and the solution was clarified by centrifugation. The resulting supernatant fraction was the acetone-precipitated material.

The acetone-precipitated material was applied directly to a chromatofocusing column and eluted with polybuffer (Pharmacia, Uppsula, Sweden). The fractions containing the

18-kDa protein were pooled and concentrated 13.3-fold by lyophilization. This sample was then applied to a Sephadex G-50 superfine column, and the fractions containing the 18-kDa protein were pooled and dialyzed. Finally, this dialyzed pool was loaded onto a DEAE-cellulose column washed and eluted as described in the legend for Figure 3C. The pooled fractions eluted with NaCl yielded the pure 18-kDa protein. About 20 g of cytosolic protein would generally yield about 30 mg of pure 18-kDa protein using this purification scheme.

Protein Sequencing and Amino Acid Analysis. The purified protein was sent to The University of Michigan protein sequencing facility (Ann Arbor, MI) for protein sequencing and amino acid analysis as described in Tarr (1986) and Hunkapillar et al. (1981).

Search of Protein Sequence Data Bases with N-Terminal Protein Sequence. The N-terminal protein sequence was used to search the National Biomedical Research Foundation (NBRF) protein data base (Release 11.0, 12/1986) for sequence homology to other known proteins.

The software used was from the University of Wisconsin Genetics Computer Group (Wilbur & Lipman, 1983; Devereux et al., 1984). The program Wordsearch compared one sequence to a group of sequences. The program found areas of the largest number of short perfect matches. The best areas of similarity were visualized with the program Segments. In all cases, the first 50 best segments were visualized.

Search of DNA Sequence Data Bases with DNA Sequence Generated from the N-Terminal Protein Sequence. A codon frequency table for the chick was generated with the DNA sequences to five chick proteins. This table and the program Backtranslate were used to generate four DNA sequences for the N-terminal protein sequence. One of these assumed all the amino acids in the protein sequence were correct and the codon frequency table was accurate. The second assumed all amino acids were correct but made no assumption for codon use where ambiguity was possible. The third and fourth were analogous to the first and second except amino acids 15, 17, and 24 were allowed to be any amino acid. These four nucleotide sequences were each used to search GenBank and the European Molecular Biology Laboratory (EMBL) DNA sequence data bases for homologous sequences in the same way that the protein sequence was used to search the protein data bases.

Assay of the 18-kDa Protein for Retinol Binding. The purified protein was diluted into 50 mM Tris-HCl, pH 7.5, and the absorbance at 280 nm was recorded. The fluorescence spectra were taken as indicated in Figure 7. Then 20.5 μL of retinol was added to 3 mL of protein solution, and the spectra were taken again. The same volume of retinol was added to 3 mL of methanol, the absorbance was measured at 325 nm, and the fluorescence spectrum was taken the same way. Fluorescence of 50 mM Tris-HCl, pH 7.5, or methanol alone was subtracted from the samples at each wavelength. The absorbance of retinol at 325 nm was used to calculate its concentration.

General. Protein concentrations were determined by using the Bio-Rad (Richmond, CA), protein microassay with ovalbumin as standard. The vitamin D dependent CaBP was purified by the method of Bishop et al. (1983).

RESULTS

Comigration of Duodenal, 18-kDa Protein from 4-Week Chick and 19-Day Embryonic Chick. The 18-kDa, 5.1 p*I* protein from 19-day embryonic chick duodena first studied by Bishop et al. (1985) comigrated exactly with a protein in 4-week chick duodena on 2D PAGE (Figures 1 and 2).

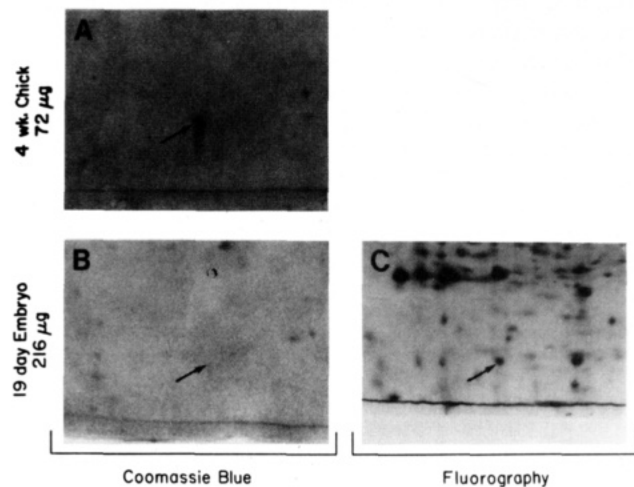

FIGURE 1: Comparison of 18-kDa protein in 4-week duodena and the protein from 19-day embryonic duodena using 2D PAGE. Identical portions of each 2D gel are shown. The arrow indicates the position of the 18-kDa protein. (A) 72 μg of 4-week chick cytosolic protein was electrophoresed, stained with Coomassie blue, and photographed. (B) 216 μg of ^{3}H-labeled embryonic proteins (6.8×10^4 dpm) was electrophoresed, stained with Coomassie blue, and photographed. (C) The gel in (B) was treated with En^{3}Hance, dried, and exposed to film for 36 days at −70 °C.

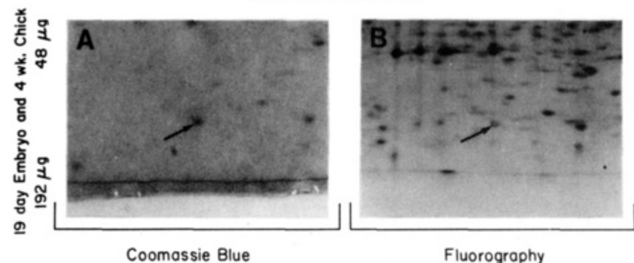

FIGURE 2: Comigration of the 18-kDa protein from 4-week-old chick duodena and radiolabeled 18-kDa protein from chick embryo duodenum. (A) 48 μg of 4-week chick cytosolic proteins was mixed with 192 μg (6×10^4 dpm) of ^{3}H-labeled embryonic chick proteins, the sample electrophoresed, and the gel stained with Coomassie blue and photographed. (B) The gel was treated with En^{3}Hance, a hole was punched in the 18-kDa/5.1 p*I* spot, and then the gel was subjected to fluorography (as in Figure 1C).

Figures 1 and 2 indicate that 48–72 μg of chick cytosol was enough to permit visualization of the 18-kDa protein with Coomassie blue stain. In fact, it is the major protein found in this preparation (Figures 1A and 2A). However, the amount of embryonic cytosol (216 μg) was insufficient to visualize the 18-kDa protein by Coomassie blue stain, demonstrating it to be in low abundance in this tissue (Figure 1B). Only fluorography of the radiolabeled proteins permitted detection of the 18-kDa protein in the embryonic cytosol (Figure 1C). When the comigration gel was stained with Coomassie blue (Figure 2A), only the protein from the chick was detected. A hole was punched in the 18-kDa spot, and the same gel was subjected to fluorography. The 18-kDa spot on the film was due only to the radioactively labeled embryonic protein. The hole placed in the Coomassie blue stained chick protein was also present in the center of the radioactive embryonic protein, demonstrating that the proteins from both sources exactly comigrate (Figure 2B).

Purification of 18-kDa Protein from 4-Week Chick Duodena. The 18-kDa protein from 4-week chick duodena was purified to homogeneity by the procedure described under

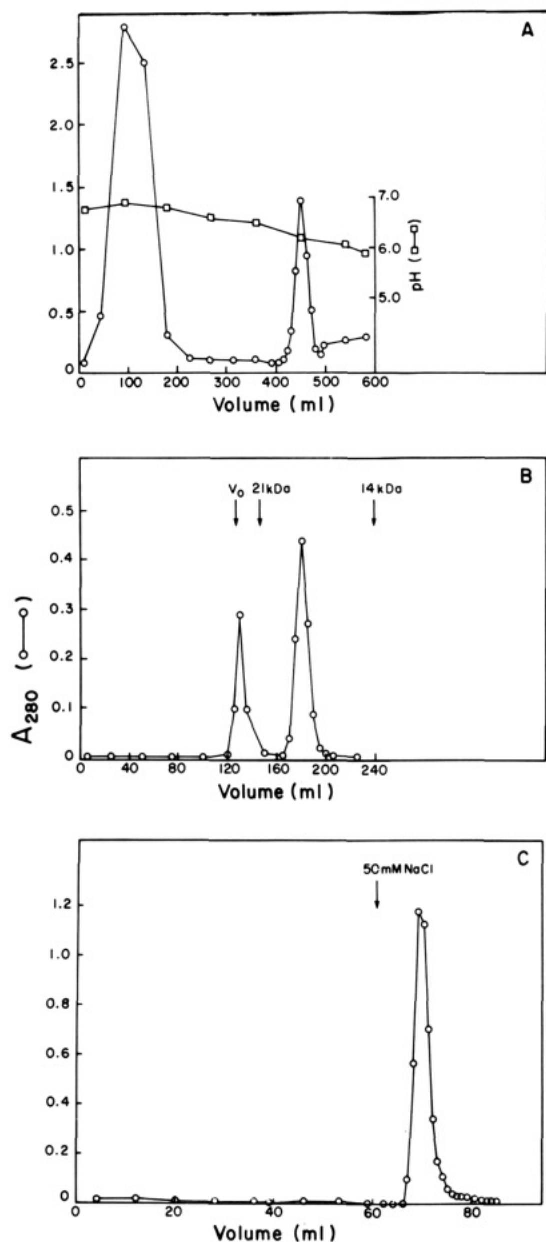

FIGURE 3: Purification of the 18-kDa protein from 4-week chick duodenal cytosol. (A) Chromatofocusing chromatography of acetone-precipitated cytosolic fraction. A column was packed with 78 mL of PBE94 chromatofocusing resin and equilibrated in 25 mM imidazole hydrochloride, pH 7.0. Then 400 mg of acetone-precipitated protein fraction was applied to the column, and the proteins were eluted with Polybuffer 74–HCl, pH 5.0, diluted 1:16. The flow rate was 26 mL/h, and 9-mL fractions were collected. Absorbance at 280 nm (○) and pH (□) were measured for the fractions, and the molecular weight of the peak fractions was determined by SDS–PAGE. Fractions from 414 to 477 mL containing the 18-kDa protein were pooled and lyophilized. (B) Gel filtration chromatography of postchromatofocusing chromatography fraction. A column was packed with 360 mL of Sephadex G-50 superfine and equilibrated with 25 mM Tris-HCl, pH 7.5. Then 7.9 mg (3.5 mL) of the reconstituted pool from chromatofocusing was applied, and 5-mL fractions were collected. Fractions were analyzed by absorbance at 280 nm and by SDS–PAGE. The fractions from 170 to 200 mL were pooled and dialyzed against 10 mM Tris-HCl, pH 7.5. (C) DEAE-cellulose chromatography of Sephadex G-50 fraction. A column was packed with 7.8 mL of DE-52 resin and equilibrated with 10 mM Tris-HCl, pH 7.5. The Sephadex G-50 fraction (7.2 mg) was loaded onto the column, washed with 60 mL of 10 mM Tris-HCl, and eluted with 10 mM Tris-HCl and 50 mM NaCl, pH 7.5. The fractions from application, washing, and elution were analyzed by absorbance at 280 nm and SDS–PAGE. Fractions from 67 to 74 mL were pooled to provide 5.5 mg of pure 18-kDa protein.

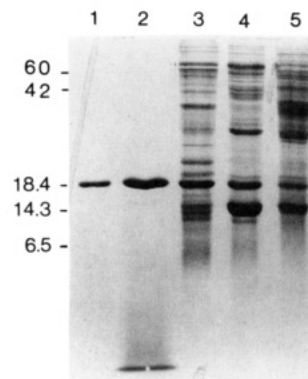

FIGURE 4: SDS–PAGE of material from each purification step. Various steps in the purification of the 18-kDa protein are described in the text. Molecular weight ($\times 10^{-3}$) is indicated. Lane 1, 1 μg of protein pooled from DEAE-cellulose chromatography; lane 2, 5 μg of protein pooled from chromatofocusing chromatography; lane 3, 20 μg of acetone-precipitated protein fraction; lane 4, 20 μg of heat-treated cytosol; lane 5, 20 μg of 4-week chick duodenal cytosol.

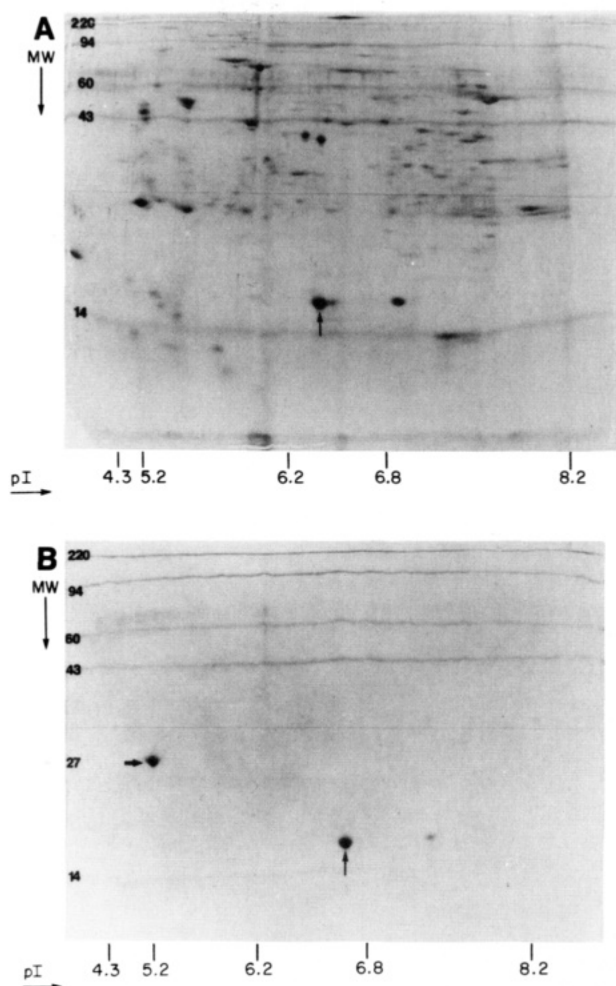

FIGURE 5: 2D PAGE of 4-week chick cytosol and purified 18-kDa protein. 2D PAGE was performed as described under Materials and Methods. The first dimension was 4% mixed IEF, and the second dimension was 12% polyacrylamide. Gels were stained with Coomassie blue. Positions of molecular weight ($\times 10^{-3}$) and p*I* markers are indicated. CaBP was electrophoresed as an internal standard with the purified 18-kDa protein and is indicated by the horizontal arrow. The 18-kDa protein is indicated by the vertical arrow. (A) 320 μg of chick duodenal cytosolic protein fraction. (B) 5 μg of 18-kDa protein from the DEAE chromatography pool.

Materials and Methods (Figure 3). SDS–PAGE or 2D PAGE was used to detect the protein after each purification step (Figures 4 and 5). After the DEAE-cellulose chroma-

Table I: Amino Acid Analysis of the 18-kDa Protein[a]

amino acid	no. of residues/18 kDa	amino acid	no. of residues/18 kDa
Ala	4.6	Leu	15.2
Cys	5.7[a]	Met	2.6
Asp	22.4	Pro	1.2
Glu	21.1	Arg	6.6
Phe	8.0	Ser	3.0
Gly	12.5	Thr	13.8
His	4.6	Val	12.9
Ile	5.5	Tyr	5.5
Lys	15.7		

[a] Cysteic acid normalized to the compositions from duplicate perfomic acid oxidized hydrolyses.

RESIDUE NUMBER

PROTEIN	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
18 kDa	P	A	D	Y	N	G	T	W	E	M	E	S	N	E	(N)	F	(E)	G	Y	M	V	A	L	(D)	I	D	F	A
CRBP II	T	K	D	Q	N	G	T	W	E	M	E	S	N	E	N	F	E	G	Y	M	K	A	L	D	I	D	F	A
CRBP	P	V	D	F	N	G	Y	W	K	M	L	S	N	E	N	F	E	E	Y	L	R	A	L	D	V	N	V	A
CRABP	P		N	F	A	G	T	W	K	M	R	S	S	E	N	F	D	E	L	L	K	A	L	G	V	N	A	M

FIGURE 6: 18-kDa protein has homology to cellular retinoid binding proteins. One milliliter of the pure 18-kDa protein pool eluted from the DEAE-cellulose column was dialyzed against 20 mM NH_4HCO_3, pH 7.8. Then 150 μg was lyophilized and sent for amino acid analysis and gas phase sequencing. Residues with uncertain identity are enclosed in parentheses. The N-terminal sequence of the 18-kDa protein was used to search the protein sequence files of the NBRF protein data bank. This was done twice, once with amino acids 15, 17, and 24 defined and once with these residues equal to any amino acid. In either case, the most homologous sequences found were CRBP II from rat intestine (Li et al., 1986), CRBP from rat liver (Rask et al., 1981), and CRABP from bovine retina (Crabb & Saari, 1981). The numbering of amino acids was the same for all N-terminal sequences except for CRABP where a gap was inserted at position 2 for alignment with the other sequences. The boxes indicate amino acids of the 18-kDa protein that are homologous in the other sequences.

tography step, the protein was homogeneous as evidenced by 2D PAGE. The purified protein had a molecular weight of 18K and a major and minor p*I* at p*I* 6.6 and 7.1, respectively. The molecular weight of 18K for the protein from SDS–PAGE is in close agreement with the molecular weight of 19K that was calculated from gel filtration data (Figure 3B). The p*I* of the protein of 6.2 determined by chromatofocusing (Figure 3A) was similar to the average p*I* of the major and minor forms. The identity was also confirmed by a comparison of the pure protein and 4-week chick cytosol by 2D PAGE analysis (Figure 5A,B). The p*I* determined for the protein was different from that of Bishop et al. (1985) because a different IEF gradient was used during 2D PAGE.

Protein Sequencing and Amino Acid Analysis. The amino acid analysis of the protein is shown in Table I. The amino acid analysis for this 18-kDa protein was similar to that which can be calculated from the DNA sequence data for CRBP II (Li et al., 1986). The 28 amino acid N-terminal protein sequence is illustrated in Figure 6.

Search of Protein Sequence Data Bases with N-Terminal Protein Sequence. The N-terminal protein sequence was used to search all protein sequences in the NBRF protein data base for homology. The N-terminal sequence of the 18-kDa protein had homology to retinoid binding proteins from several sources and also to bovine and murine myelin P2 protein (Kitamura et al., 1980). The protein had the most silmilarity to the N-terminal sequence recently published for CRBP II (Li et al., 1986). The N-terminal sequence for the 18-kDa protein and its three closest homologues is illustrated in figure 6.

Search of DNA Sequence Data Bases with DNA Sequences of the 18-kDa N-Terminus. The DNA sequences generated from the 18-kDa protein N-terminal sequence were used to search the GenBank and EMBL data bases for homology. The best match was to the rabbit Ig κ chain although there was slight homology found to regions of human Ig heavy chains.

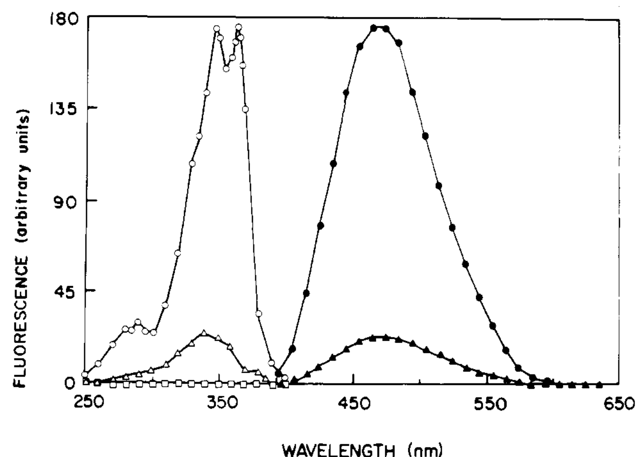

FIGURE 7: Purified 18-kDa protein binds to retinol. Excitation spectra were measured from 250 to 400 nm monitoring emission at 470 nm. Emission spectra were monitored from 375 to 625 nm with excitation at 348 nm. Excitation (○) and emission (●) spectra of 18-kDa protein, absorbance at 280 nm = 0.170, and 1.23 × 10^{-5} M retinol in 50 mM Tris-HCl, pH 7.5. Excitation spectrum of the 18-kDa protein, absorbance at 280 nm = 0.170, in 50 mM Tris-HCl, pH 7.5 (□). Excitation (△) and emission (▲) spectra of 1.23 × 10^{-5} M retinol in methanol.

Assay of the 18-kDa Protein for Retinol Binding. The purified protein was assayed for retinol binding activity by fluorescence. The purified protein had no fluorescence and retinol very little fluorescence when excitation and emission spectra were taken as indicated in Figure 7. When retinol and the 18-kDa protein were combined, there was a large increase in both the excitation and emission maxima. The excitation spectrum of the 18-kDa protein and retinol was complex with three maxima at 290, 348, and 364 nm. The emission spectrum was a smooth curve with a maximum at 465 nm.

DISCUSSION

Our approach to the study of this 1,25-(OH)$_2$D$_3$-modulated protein was to purify and characterize it. We observed that relatively small amounts of the protein are present in the embryonic intestine but that an abundant protein in 4-week chick intestine migrated in a similar position during 2D PAGE. Our comigration experiment (Figures 1 and 2) showed that the protein from 4-week chick intestine is the same protein as the low-abundance 1,25-(OH)$_2$D$_3$-modulated protein from embryonic intestine. Therefore, we used the 4-week chick intestine as a source of the protein. The protein was deemed homogeneous after our purification procedure on the basis of SDS–PAGE and 2D PAGE analysis (Figures 4 and 5).

The purified protein had a major and a minor p*I* form as evidenced by 2D PAGE (Figure 5B). When the sample containing the two forms (Figure 5B) was sequenced, a single unambiguous sequence was obtained, indicating that the two proteins were the same but probably just contain a small amount of charge heterogeneity C-terminal to the first 28 amino acids.

We obtained the N-terminal sequence of the protein so that it would be possible to determine the identity of the protein by using existing protein and DNA sequence data bases. In both the GenBank and EMBL DNA sequencc data bases, the most homologous sequence for all four generated nucleotide sequences to the 18-kDa protein was to the Ig κ light chain from rabbit. There was also some homology to regions of

human Ig heavy chains. The significance of this is difficult to assess at this time.

We found that the protein had the most significant homology to CRBP II, an intestinal, 16-kDa protein with *all-trans*-retinol as its endogenous ligand (Ong, 1984). Immunohistochemical studies of the rat indicate that CRBP II exists primarily in the absorptive cells of the small intestine, implying a role for the protein in intestinal absorption (Crow & Ong, 1985). Further work by Ong et al. (1987) indicates that this protein may function in presenting absorbed retinol to the correct esterifying enzyme in the intestine. The primary structure for CRBP II has been determined, and it has also been shown that the mRNA for the protein is highest in the intestine and that fetal mRNA concentrations are higher than in the adult (Li et al., 1986).

The strong sequence homology, 24 out of 28 N-terminal amino acids, and identical organ localization provided evidence that the 18-kDa protein might be CRBP II. The strong sequence homology was especially convincing since the 18-kDa protein was from the chicken and the CRBP II sequence is from the rat. More differences would be expected between the sequences of avian and mammalian proteins unless they are conserved evolutionarily. The similar amino acid analysis for both proteins also suggested that the homology continues in the remainder of the peptide chain.

Finally, the fluorescence data (Figure 7) indicated that the pure 18-kDa protein bound retinol and gave an excitation spectrum characteristic of CRBP II–retinol interaction. Both the 18-kDa protein and retinol have little fluorescence alone, but a large increase was seen when a complex was formed between the two molecules. The excitation and emission spectra of the 18-kDa protein–retinol complex were strikingly similar to the spectra recorded by Ong (1984) for the CRBP II–retinol complex. Both proteins, when combined with retinol, give a complex excitation spectrum with three maxima and a smooth emission spectrum with one maximum. The wavelengths of these four maxima were almost identical between the two proteins.

All of the above provide strong evidence that the 18-kDa protein we have been studying is CRBP II. Immunological properties, physical characteristics, and total sequence of the 18-kDa protein and CRBP II will have to be compared under identical conditions before identity of the 18-kDa protein and CRBP II can be confirmed. Some differences might be expected between the avian and mammalian proteins.

Previous work indicates that there is some interaction between vitamins A and D at the biochemical level. There is evidence that vitamin A and its active metabolites can modulate 1,25-$(OH)_2D_3$ receptor binding activity (Petkovich et al., 1984) and 25-OH-D_3 1- and 24-hydroxylase activities (Trechsel & Fleisch, 1981). There are also reports that the toxic effects due to high intakes of either vitamin can be alleviated by intake of the other vitamin (Veltmann et al., 1987). The link between vitamin A activity and vitamin D activity could possibly be explained if CRBP II, a protein apparently involved in esterification of retinol, is modulated by 1,25-$(OH)_2D_3$.

It is interesting to note that the 18-kDa protein is one of the most abundant proteins in chicken cytosol yet there was so little of the protein in the embryo cytosol that fluorography is needed to detect it. Although CRBP II appears to be more abundant in the fetal than adult rat, these data may be consistent with ours since there is a rapid fluctuation in the amount of protein right at the time of birth (Li et al., 1986). The 1,25-$(OH)_2D_3$-dependent modification is observed when the embryos are 19 days old, before their intestines actually take up calcium. It is possible that the concentration of the protein in the embryonic chick could change dramatically around the 19-day period. Normally, the chick does not hatch until day 21 so that this 19-day period may be a crucial time when the embryo is switching from obtaining calcium from the shell through the chorioallantoic membrane to obtaining calcium from food through the intestine.

In summary, we have shown that the 18-kDa protein modulated by 1,25-$(OH)_2D_3$ may be CRBP II. We have worked out a method for obtaining milligram quantities of the pure protein and carried out its initial characterization. Work in our laboratory is ongoing to perform experiments to study the disappearance of the 18-kDa protein in response to 1,25-$(OH)_2D_3$. Determining the nature of this disappearance and the mechanism whereby 1,25-$(OH)_2D_3$ induces it may contribute to an understanding of the link between vitamin A and D actions and how 1,25-$(OH)_2D_3$ stimulates calcium and phosphate transport.

Registry No. *all-trans*-Retinol, 68-26-8.

References

Bishop, C. W., Kendrick, N. C., & DeLuca, H. F. (1983) *J. Biol. Chem. 258*, 1305–1310.

Bishop, C. W., Kenrick, N. C., & DeLuca, H. F. (1984) *J. Biol. Chem. 259*, 3355–3360.

Bishop, C. W., Kendrick, N. C., Dame, M. C., & DeLuca, H. F. (1985) *J. Biol. Chem. 260*, 5209–5212

Bredderman, P., & Wasserman, R. H. (1964) *Biochemistry 13*, 1687–1694.

Corradino, R. A. (1973) *Nature (London) 243*, 41–43.

Crabb, J. W., & Saari, J. C. (1981) *FEBS Lett. 130*, 15–18.

Crow, J. A., & Ong, D. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4704–4711.

DeLuca, H. F. (1986) in *Steroid Hormone Resistance. Mechanisms and Clinical Aspects* (Chrousos, G. P., Loriaux, D. L., & Lipsett, M. B., Eds.) pp 361–375, Plenum, New York.

DeLuca, H. F., & Schnoes, H. K. (1984) in *Annual Reports in Medicinal Chemistry* (Bailey, D. M., Ed.) pp 179–190, Academic, New York.

Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387–395.

Franceschi, R. T., & DeLuca, H. F. (1981a) *J. Biol. Chem. 256*, 3848–3852.

Franceschi, R. T., & DeLuca, H. F. (1981b) *J. Biol. Chem. 256*, 3840–3847.

Harmeyer, J., & DeLuca, H. F. (1969) *Arch. Biochem. Biophys. 1338*, 247–254.

Hart, L. E., DeLuca, H. F., Yamada, S., & Takayama, H. (1984) *J. Nutr. 114*, 2059–2065.

Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Kitamura, K., Suzuki, M., Suzuki, A., & Uyema, K. (1980) *FEBS Lett. 115*, 27–30.

Li, E., Demmer, L. A., Sweetser, D. A., Ong, D. E., & Gordon, J. I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5779–5783.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4020.

Ong, D. E. (1984) *J. Biol. Chem. 259*, 1476–1482.

Ong, D. E., Kakkad, B., & MacDonald, P. N. (1987) *J. Biol. Chem. 262*, 2729–2736.

Petkovich, P. M., Hursche, J. N. M., Tinker, D. O., & Jones, G. (1984) *J. Biol. Chem. 259*, 8274–8280.

Rask, L., Anundi, H., Bohme, J., Ericksson, U., Ronne, H., Sege, K., & Peterson, P. A. (1981) *Ann. N.Y. Acad. Sci. 359*, 79–90.

Spencer, R., Charman, M., Wilson, P., & Lawson, E. (1976) *Nature (London) 263*, 161–163.
Tarr, G. E. (1986) in *Microcharacterization of Polypeptides: A Practical Manual* (Shively, J. E., Ed.) pp 155–194, Humana, Clifton, NJ.
Trechsel, U., & Fleisch, H. (1981) *FEBS Lett. 135*, 115–118.
Veltmann, J. R., Jr., Jensen, L. S., & Rowland, G. N. (1987) *Nutr. Rep. Int. 35*, 381–392.
Wilbur, W. J., & Lipman, D. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 726–730.

^{1}H Fourier Transform NMR Studies of Insulin: Coordination of Ca^{2+} to the Glu(B13) Site Drives Hexamer Assembly and Induces a Conformation Change[†]

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ABSTRACT: ^{1}H Fourier transform NMR investigations of metal ion binding to insulin in ^2H_2O were undertaken as a function of pH* to determine the effects of metal ion coordination to the Glu(B13) site on the assembly and structure of the insulin hexamer. The C-2 histidyl regions of the ^{1}H NMR spectra of insulin species containing respectively one Ca^{2+} and two Zn^{2+}/hexamer and three Cd^{2+}/hexamer have been assigned. Both the Cd^{2+} derivative $(In)_6(Cd^{2+})_2Cd^{2+}$, where two of the Cd^{2+} ions are coordinated to the His(B10) sites and the remaining Cd^{2+} ion is coordinated to the Glu(B13) site [Sudmeier, J. L., Bell, S. J., Storm, M. C., & Dunn, M. F. (1981) *Science (Washington, D.C.) 212*, 560], and the Zn^{2+}–Ca^{2+} derivative $(In)_6(Zn^{2+})_2Ca^{2+}$, where the two Zn^{2+} ions are coordinated to the His(B10) sites and Ca^{2+} ion is coordinated to the Glu(B13) site, give spectra in which the C-2 proton resonances of His(B10) are shifted upfield relative to metal-free insulin. Spectra of insulin solutions (3–20 mg/mL) containing a ratio of In:Zn^{2+} = 6:2 in the pH* region from 8.6 to 10 were found to contain signals both from metal-free insulin species and from the 2Zn–insulin hexamer, $(In)_6(Zn^{2+})_2$. The addition of either Ca^{2+} (in the ratio In:Zn^{2+}:Ca^{2+} = 6:2:1) or 40 mM NaSCN was found to provide sufficient additional thermodynamic drive to bring about the nearly complete assembly of insulin hexamers. Cd^{2+} in the ratio In:Cd^{2+} = 6:3 also drives hexamer assembly to completion. We postulate that the additional thermodynamic drive provided by Ca^{2+} and Cd^{2+} is due to coordination of these metal ions to the Glu(B13) carboxylates of the hexamer. At high pH*, this coordination neutralizes the repulsive Coulombic interactions betwen the six Glu(B13) carboxylates and forms metal ion "cross-links" across the dimer–dimer interfaces. Comparison of the aromatic regions of the ^{1}H NMR spectra for $(In)_6(Zn^{2+})_2$ with $(In)_6(Zn^{2+})_2Ca^{2+}$, $(In)_6(Cd^{2+})_2Cd^{2+}$, and $(In)_6(Cd^{2+})_2Ca^{2+}$ indicates that binding of either Ca^{2+} or Cd^{2+} to the Glu(B13) site induces a conformation change that perturbs the environments of the side chains of several of the aromatic residues in the insulin structure. Since these residues lie on the monomer–monomer and dimer–dimer subunit interfaces, we conclude that the conformation change includes small changes in the subunit interfaces that alter the microenvironments of the aromatic rings.

Insulin is synthesized and stored in the secretory granules of the pancreatic β-cells as a crystalline array of hexamers (Lacy, 1957; Greider et al., 1969; Howell, 1974). The high concentrations of both Zn^{2+} and Ca^{2+} found in these granules (Hellman et al., 1976; Howell et al., 1978; Anderson & Berggren, 1979) and recent studies (Sudmeier et al., 1981; Storm & Dunn, 1985; Dunn et al., 1987; Kaarsholm & Dunn, 1987) strongly suggest these hexamers contain both ions bound to specific sites on the insulin hexamer.

The X-ray diffraction studies of insulin carried out independently in three different countries (Blundell et al., 1972; Bentley et al., 1979; Cutfield et al., 1979, 1981; Peking Insulin Structure Research Group, 1974; Sakabe et al., 1981; Dodson et al., 1979, 1980) have provided refined, high-resolution structures for four different forms of insulin. These forms are respectively the porcine 2Zn–insulin hexamer (Peking Insulin Structure Research Group, 1974; Sakabe et al., 1981; Dodson et al., 1979, 1980; Cutfield et al., 1981; Baker et al., 1987), the porcine 4Zn–insulin hexamer (Bentley et al., 1976; Cutfield et al., 1981; Chothia et al., 1983; Smith et al., 1984), the hagfish metal-free insulin dimer (Cutfield et al., 1979, 1981), and a chemically modified monomeric insulin (Bi et al., 1984; Dong-Cai et al., 1983).

Both the 2Zn and 4Zn hexamers are torus-shaped molecules (Chart I). The insulin subunits are arranged in each hexamer as three symmetry-related insulin dimers about the threefold axis. Because each dimer is asymmetric in the crystal, there are two different subunit conformations in each hexamer. In the 2Zn structure (Chart I), the conformational differences that give rise to this asymmetry are small. In the 4Zn structure, the conformational differences are large: one subunit retains a conformation almost identical with that found in 2Zn–insulin and the other subunit had a large change in the

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