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The Purification And Properties Of An Intestinal Calcium Binding Protein From The Baboon

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In contrast, Wecksler and Norman, (1979) and Wecksler and Norman, (1980) and more recently Haussler et al, (1981) presented data that the chromatin binding step was rapid compared to the formation of the steroid-receptor complex and was not greatly influenced by relatively small changes in temperature (0 - 23^{o}C). It thus seems that the apparent effects of temperature on chromatin binding were primarily affecting the formation and dissociation of steroid to the receptor (Bikle et al, 1981).

One of the most important contributions in the field of vitamin D research was the discovery of an intestinal CaBP (Wasserman and Taylor, 1966), a protein which was originally hypothesised to be involved in transporting calcium across the cell. Administration of cholecalciferol to rachitic chicks caused an increase in the calcium binding activity which was associated with a protein of molecular weight of 28 000 daltons. It was suggested that this protein possibly functions to facilitate the removal of calcium from the cell by mediating a transfer of calcium from the mitochondria (or other subcellular organelles transporting calcium through the cell) to the calcium pump in the basolateral membrane.

To date, a number of vitamin D-dependent CaBPs have been characterised from the intestinal mucosa, kidney, brain, bone, pancreas and also placenta of a variety of lower vertebrates, avian and mammalian species. Research conducted on the vitamin D-dependent CaBP includes tissue distribution, biochemical properties, vitamin D-dependency, cellular and subcellular localisation and molecular biology.

1.2 Reptilian CaBPs

This group of vertebrates has only been studied briefly with respect to CaBPs. Reptiles are phylogenetically an important group that made an initial transition from the anamniotic lower vertebrates to the amniotic condition of the higher vertebrates. Like other animals (birds and mammals) the adult reptilian kidney arises from metanephrogenic mesenchyme and consists of paired secretory organs (Fox, 1977).

Although the rat renal (mammalian) and chick intestinal (avian) CaBP have a molecular weight of 28 000 daltons, a comparison of the amino acid compositions of these proteins indicates they are similar but not identical (Pansini and Christakos, 1984). Immunologically the proteins are similar and in order to obtain information on the phylesis of these closely related CaBPs, a study on the reptile the Green anole, was carried out (Rhoten et al, 1984). A CaBP with the apparent molecular weight of 28 000 daltons was localised in the kidney of Anolis carolensis using antisera directed against vitamin D-dependent CaBP from either rat kidney or chick intestine. When extracts of female saurian kidneys were fractionated by gel filtration on Sephadex G-100, both calcium binding activity and CaBP immuno-reactivity, as measured by radio-immunoassay for rat CaBP, were observed near the 28 000 dalton region, similar to chick CaBP. Utilization of the immunoblot technique following SDS acrylamide gel electrophoresis resulted in cross-reactivity of 28 000 dalton CaBP for both rat and anolian kidneys.

Immuno-reactive CaBP was localised in the nephron using the unlabelled antibody peroxidase-anti-peroxidase technique. Distal tubules gave a strong specific reaction with either antiserum but not all cells

of the distal tubule reacted with equal intensity. Cells in the kidney interstitial space of the medulla between the distal tubule and the terminal tubules and the cells in the collecting tubules were only occasionally positive. Renal corpuscles, proximal tubules and thin segments gave no specific localisation for CaBP. The sexual segment of male kidneys was also negative. These results indicate that a CaBP with an apparent molecular weight of 28 000 dalton is highly conserved during vertebrate evolution. The localisation of the CaBP to the distal tubule suggests that this protein may be involved in the selective reabsorption and/or secretion of calcium.

1.3 Avian CaBPs

Administration of vitamin D to the chick was shown to lead to the appearance of a CaBP in the small intestinal mucosa and kidney (Wasserman and Taylor, 1966; Taylor and Wasserman,1967). The appearance of the intestinal protein was highly correlated with the intestinal localisation of $1,25(OH)_2D_3$ (Friedlander et al, 1977). The vitamin D-dependency of the chick CaBP has been reported in many studies (Norman et al, 1982). Proteins immunologically indistinguishable from chick intestinal CaBP have been found in 8 tissues (see Table I).

Tissues other than the intestinal mucosa have been studied, detection being by immunocytochemical techniques.

TABLE I Tissue Distribution and Molecular weights of Chick vitamin D-dependent CaBPs

Chick	Reported Molecular Weight (daltons)
Intestine	28 000[1]
Kidney	28 000[2]
Bone	34 000[3]
Pancreas	28 000[4]
Brain	28 000[5]
Parathyroid	N.D.[6]
Egg shell gland	N.D.[7]
Chorioallantoic Membrane	N.D.[8]

N.D. not determined

1) (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967).

2) (Christakos and Norman, 1980).

3) (Christakos and Norman, 1978).

4) (Morrissey, 1975; Kadowaki et al, 1982; Roth et al, 1982a).

5) (Taylor, 1977; Jande et al, 1980, 1981; Roth et al, 1981a, b).

6) (Christakos et al, 1979b).

7) (Corradino et al, 1968; Lippiello and Wasserman, 1975; Jande et al, 1981).

8) (Tuan et al, 1978; Coty et al, 1980).

It is evident from the table that the molecular weight of all the CaBPs in all tissues in the chick other than bone is 28 000 daltons. The partially purified bone CaBP was found to have a molecular weight of 34 000 daltons and was also shown to be vitamin D-dependent (Chistakos and Norman, 1978). The difference in the molecular weight suggests that bone CaBP is different to intestinal CaBP. The bone CaBP is concentrated in the spongiosa and cartilage plate regions of the tibia and responds adaptively to reflect the level of dietary calcium.

The biochemical properties of the vitamin D-dependent CaBPs of the chick have been well characterised. The association constant of the CaBP - Ca complex was found to be 2,6 X $10^{5}M^{-1}$ (Wasserman et al, 1968). It was later shown to be 2 X $10^{6}M^{-1}$ for high affinity binding sites (Bredderman and Wasserman, 1974). The difference occurred in that both n and k_i for calcium binding to the high affinity sites of CaBP differ from the values previously reported by Wasserman et al, (1968), obtained by ion-exchange data using the method and equation of Schubert et al, (1950). This 8-fold difference was revealed after a re-examination of the Schubert equation. It was shown mathematically that it is not applicable to Ca binding to have a set of identical, independent sites on a protein. (Bredderman and Wasserman, 1974). The relative binding affinities for alkaline earths follows the same order as that of citrate and EDTA i.e. calcium > strontium > barium (Wasserman, 1962). The nature of the binding site was unknown, but studies showed that the binding activity was dependent on both ionic strength and pH. Analysis of the binding data showed that the chick protein binds four Ca ions per molecule (Bredderman and Wasserman 1974).

The purified chick CaBP was found to be free from lipids, carbohydrates and phosphorous (Bredderman and Wasserman, 1974). These authors found the molecular weight of the intestinal protein by amino acid analysis to be 27 000 daltons and reported a high content of dicarboxylic acids (30%). The high proportion of these acids was also found in hydrolysates of troponin A (33%) and other high affinity CaBPs (Ebashi et al, 1971; Sottacassa et al, 1972; Nockolds et al, 1972; Fullmer and Wasserman, 1973). The isoelectric point was calculated to be 4,2 and was shown by Ingersoll and Wasserman, (1971) to be 4,2 - 4,3. On the basis of amino acid composition the partial specific volume of the protein was calculated to be 0,734 g/cm^3 and the average charge per residue to be 0,384 (Bredderman and Wasserman, 1974).

Circular dichroism measurements of the chick CaBP showed that it had only 30 - 40% α-helicity and underwent only a small change in helical content when calcium bound (Ingersoll and Wasserman, 1971). The fact that high affinity calcium binding is destroyed by chemical denaturants suggests that it is bound in a polydentate chelate complex (Ingersoll and Wasserman, 1971). High thermal stability (60° and higher) is a feature of the intestinal CaBP of all species thus far examined (Hitchman and Harrison, 1972).

A study on the thermal stability of the chick intestinal CaBP indicated that its immuno-reactivity, high affinity binding for calcium and electrophoretic mobility are unchanged up to a temperature of $80^{\circ}C$ (Bredderman and Wasserman, 1974). From $80^{\circ}C$ onward the stability declined precipitously. No time course studies were conducted to determine the length of time these parameters could be measured at the different temperatures studied, particularly at $80^{\circ}C$.

The chick CaBP was detected in all segments of the small intestine and kidney of rachitic chicks fed vitamin D_3. However, the macromolecule was not detected at the time in colon, liver or muscle. Thus, it was suggested that the soft tissue localisation of the CaBP represented known physiological sites of vitamin D_3 action. In the small intestine the concentration of the CaBP in vitamin D_3 replete chicks decreased in the order of the duodenum > jejunum > ileum which correlated with the relative abilities of these particular segments to absorb calcium (Wasserman et al, 1968).

A vitamin D-dependent CaBP in the chick kidney, localised exclusively in the distal convoluted tubules was found (Roth et al, 1981c). Their results indicated that there might well be a role for the vitamin D-dependent CaBP as part of an intracellular transport mechanism, rather than a direct involvement in membrane-mediated calcium reabsorption in the avian kidney. The CaBP was found also to be in the initial collecting tubule as well as the early part of the collecting tubule.

A CaBP was detected in the chick brain and its properties were compared with that of chick intestinal CaBP, (Roth et al, 1981 a, b). It was found that the two proteins were immunologically identical, as were their electrophoretic mobilities. The concentration of the brain CaBP was highest in the cerebellum. Unlike the chick intestinal CaBP, the cerebellar form displayed no dependence on vitamin D_3 or $1,25(OH)_2D_3$. The molecular weight of the protein was found to be 28 000 daltons (Taylor, 1974).

Vitamin D-dependent CaBP has also been detected by immunocytochemistry in the chick pancreas, exclusively in the ß-cells (Roth et al, 1982a). The identification of the pancreas as a target organ was a surprising find. However, it was demonstrated that vitamin D-deficiency inhibited pancreatic secretion of insulin (Norman et al, 1980). This inhibition could be explained in that both $1,25(OH)_2D_3$ and CaBP are associated with the endocrine function, most probably in the ß-cell, or at the site of insulin secretion.

Thus in both the chick brain, where CaBP can be considered to be a neutral marker, and in the pancreas, where CaBP is found only in the β-cells, a role for CaBP has not yet been established. However, it is known that calcium has an effect on several CNS functions and that in the pancreas vitamin D status affects insulin secretion. Furthermore, calcium is known to play an important role in insulin release, and the localisation of CaBP in the β- cells favours a relationship between the action of vitamin D metabolites, calcium metabolism and β-cell function. (Norman, et al, 1982).

Recently the biochemical properties of the chick pancreatic CaBP and its response to vitamin D-status and dietary calcium and phosphate levels were studied by Kadowaki and Norman, (1984). The authors compared their results with known vitamin D-dependent CaBPs present in the chick intestine and kidney. The pancreatic CaBP was shown to be homologous to the chick intestinal CaBP on the basis of immunological cross-reactivity, melecular size (28 200 daltons) and charge properties (chromatographic mobility on DEAE - Sephadex in the presence of either EDTA or calcium). Pancreatic levels of CaBP responded to changes in vitamin D-status and dietary calcium and phosphate levels in a similar way to the intestinal

CaBP. Thus, in the absence of dietary vitamin D, both pancreatic and intestinal CaBPs were essentially undetectable, while in the presence of dietary vitamin D, a low dietary phosphate (0,05%) elevated the pancreatic and intestinal CaBPs 1,5 times and 1,6 times respectively, compared to CaBP levels present with normal dietary Ca and P (1,0%, 1,0%). Collectively, these data suggest pancreatic CaBP has an homologous function to intestinal CaBP, and may be involved in the regulation of islet cellular calcium metabolism, which is known to play an important role in insulin secretion.

It has been reported that the highest concentration of CaBP is present in the duodenum in the bird and mammal. The CaBP can constitute up to 1 - 3% of the soluble protein associated with the cell (Norman et al, 1982). As reported by Christakos et al, (1979a) tissues with the second and third highest concentration of the CaBP were the kidney and pancreas. When corrections were made for the pancreatic islet - ß - Cell distribution and the exclusive localisation of the CaBP therein, the calculated concentration of the CaBP was as much as 30% of that present in epithelial cells (Kadowaki et al, 1982).

Studies to demonstrate the involvement of CaBP in the intestinal calcium transport mechanism have been carried out (Corradino et al, 1976). Vitamin D_3 or $1,25(OH)_2D_3$ induced CaBP synthesis and stimulated transmucosal calcium transport in embryonic chick duodena maintained in an agar culture apparatus. When added to a sterol-free culture medium, highly purified chick intestinal CaBP specifically stimulated calcium transport into the cultured duodena. The authors felt that despite the specific CaBP stimulation of calcium transport, there were two reasons for exercising caution in the interpretation of the their results.

The first reason was that there exists limited knowledge of the kinetics of CaBP - 'reconstituted' calcium transport and secondly, the localisation of exogenously supplied CaBP during stimulation of calcium transport was unknown. It was with these reservations, that the authors concluded that the vitamin D-induced CaBP is involved in intestinal calcium transport and may in fact be some sort of calcium transport protein (Corradino et al, 1976).

Norman and Leathers, (1982) prepared a photoaffinity probe for vitamin D-dependent chick intestinal CaBP. This was done by conjugating methyl-4-azido benzoimidate (MABI) to iodinated (lactoperoxidase) - ^{125}I - CaBP to yield ^{125}I - CaBP - MABI. After incubation of ^{125}I - CaBP - MABI (28 000 daltons) with bovine intestinal alkaline phosphatase (68 000 daltons) in vitro, a U.V. light-induced cross linking occurred to yield a complex with a molecular weight of 95 000 daltons (as shown by SDS electrophoresis). The formation of this ^{125}I - CaBP - MABI - AP was found to occur only in the presence of calcium ions. Dietary vitamin D and $1,25(OH)_2D_3$ are known to increase the activity of intestinal brush border membrane-bound alkaline phosphatase. The significance of this result shows that a specific calcium dependent interaction exists with bovine alkaline phosphatase. This is suggestive that calcium may mediate conformational changes in the CaBP molecule which facilitates the interaction of the CaBP with cellular constituents.

The effects of dietary calcium and phosphate on the relationship between $25-(OH)D_3$-1-hydroxylase and the production of the chick intestinal CaBP was studied (Friedlander et al, 1977). These authors showed that in the presence of dietary vitamin D an incremental fall of serum calcium from 10 - 5mg/100ml resulted in a 9-fold elevation of the

1-hydroxylase and an increase in CaBP from 15 - 60 µg/mg of protein. A fall in serum phosphate from 7 - 2 mg/100ml resulted in only a 2,5-fold increase in the 1-hydroxylase, while CaBP levels increased to 45 µg/mg of protein. The levels of ^{3}H-labelled $1,25(OH)_2D_3$ isolated from the intestinal mucosa following ^{3}H-labelled $1,25(OH)_2D_3$ administration was found to be directly related to the level of intestinal CaBP over the range of calcium and phosphate concentrations used. The specific activity of the renal 25-hydroxyvitamin D_3 - 24 - hydroxylase was also investigated.

There were no identifiable correlations throughout the prevailing range of serum calcium and phosphate levels. Furthermore, the levels only varied from 0,07 to 0,25pmol/min/mg of protein. Levels of intestinal CaBP were also measured in chickens raised under similar dietary conditions and which had been only administered $1,25(OH)_2D_3$. The adaptive response due to the varying calcium content of the diet was entirely eliminated, while that resulting from the phosphate content was retained. This result was irrespective of the dose of $1,25(OH)_2D_3$ given to the birds.

This suggests that adaptive changes to phosphate are not mediated principally through the renal 1-hydroxylase. Thus it was proposed that the stimulation of calcium absorption by low dietary calcium and phosphate induced two similar endocrine control mechanisms. Stimulation of calcium absorption by low dietary calcium was induced by an increase in the production of $1,25(OH)_2D_3$, by renal 1-hydroxylase. This resulted in an increased accumulation of $1,25(OH)_2D_3$ through an unknown mechanism, which is not completely dependent on increased 1-hydroxylase activity (Friedlander et al, 1977).

The relationship between intestinal and plasma CaBPs and calcium absorption in the chick have been studied (Bar et al, 1979). Studies were conducted to assess the relationship of duodenal CaBP of cholecalciferol fed chicks maintained on various dietary calcium intakes, to calcium absorption capacity or to the amount of calcium absorbed in vivo. In addition to this, the possible relationship between duodenal and blood plasma CaBP was evaluated.

Results obtained showed that plasma calcium increased as dietary calcium intake increased (Bar et al, 1979). Plasma CaBP concentration of cholecalciferol-fed chicks was within the range 15-56 ng/ml and the duodenal and plasma CaBP concentrations of cholecalciferol deficient chicks were 21 $\pm$ 4 µg/g and 3,3 $\pm$ 0,3 ng/ml respectively. Similarly the duodenal CaBP decreased as dietary calcium increased. The total daily amount of absorbed calcium increased with dietary intake of up to 800 mg/day, while the percentage calcium absorption decreased. Thus, duodenal CaBP and plasma CaBP were positively correlated to the percentage of net calcium absorption and negatively correlated to daily amount of calcium absorbed.

Vitamin D has been shown to regulate calcium transport by specific mechanisms. After administration of $1,25(OH)_2D_3$ to vitamin D-deficient animals it was shown that, within two hours, the permeability of the intestinal brush border membrane to calcium increased (Bikle et al 1981). This resulted in an influx of calcium into the cell. This rise in intracellular calcium paralleled the initial increase in trancellular calcium transport. The calcium in the cell accumulated primarily in the mitochondria. After 8 hours, the intracellular calcium concentration declined, and reached prestimulated levels after a further 16 hours i.e.

at 24 hours. This occurred despite the continuing increases in transcellular calcium transport. Inhibition of protein synthesis failed to block either initial increases in intracellular calcium or the stimulation of calcium transport following $1,25(OH)_2D_3$ administration. The subsequent decline in intracellular calcium did not occur when protein synthesis was inhibited (Bikle, 1983).

These results can be interpreted to mean that the initial actions of $1,25(OH)_2D_3$ to increased calcium entry at the brush border and the accumulation of calcium by the mitochondria do not need new protein synthesis. However, stimulation by $1,25(OH)_2D_3$ of the movement of calcium through and from the cell at the basolateral membrane might require protein synthesis. The mechanism by which $1,25(OH)_2D_3$ enhances calcium efflux at the basolateral membrane of the cell is not clear, but it probably involves the effect of a Ca-ATPase (Van Os and Ghijsen, 1982). Therefore, $1,25(OH)_2D_3$ acts on the cell in part by receptor mediated changes in protein synthesis and in part by actions not requiring new protein synthesis.

A study was carried out by Shinki et al, (1982) in which a possible role for CaBP induction by $1,25(OH)_2D_3$ in the intestinal calcium transport system was investigated. Results obtained showed there was a relationship between calcium absorption and CaBP synthesis. These results suggested that CaBP was not necesssary in initiating calcium transport, but that it plays an important role in the maintenance of an enhanced transport mechanism. This relationship suggested that CaBP played an important role in the interaction of the calcium transport across the intestinal mucosa.

Calcium entry across the brush border membrane into the cell does not require the expenditure of energy but occurs down a steep electrochemical gradient (Rasmussen, 1979; Miller and Bronner, 1981). The lipid composition of the brush border has a high cholesterol : phospholipid ratio which results in a high viscosity of the membrane. Decreases in the membrane viscosity by an increase in temperature or unsaturated fatty acid content, increases the permeability of the brush border membrane to calcium (Schachter and Schinitzky, 1977; Bikle et al, 1982). These data suggested that one mechanism controlling calcium permeability is the lipid composition of the membrane. However, there is evidence for a calcium carrier mediating calcium movement through the brush border membrane. A CaBP has been identified in the brush border membrane which could serve as a calcium carrier (Schachter and Kowarski, 1982). Most likely, both mechanisms, lipid modulation of membrane permeability to calcium and protein-mediated calcium movement play roles in regulating calcium across the brush barder.

Efforts to elucidate the mechanism of stimulation of intestinal calcium transport have been focused on evaluating the protein composition and topology of the brush border membrane and its associated core material. A number of studies suggested that at least one site of action is the intestinal brush border membrane which results in the increase in the permeability of this membrane to calcium (Schachter and Rosen, 1959; Harrison and Harrison, 1960, 1965; Kimberg et al, 1961; Adams and Norman, 1970; Wong et al, 1970; Wong and Norman, 1975).

The composition, structure, component interactions and potential notaly regulation of the chick intestinal brush border membrane proteins which are associated not only with the lipid bilayer but also with the core material has been extensively studied by a number of researchers

(Staehelin, 1971; Rodewald et al, 1976; Mooseker, 1976; Bretscher and Weber, 1978; Craig and Powell, 1980; Mukherjee and Mooseker et al, 1980; Glenney et al, 1981; Howe et al, 1980).

Recently, studies on the effect of vitamin D on the protein composition and core material structure of the chick intestinal brush border membrane were conducted (Putkey and Norman, 1983). Vitamin D_3 is known to stimulate the absorption of calcium across the asymmetric intestinal epithelial cells. Intestinal brush border membranes were isolated from vitamin D-replete and deficient chicks. Core material proteins were isolated from brush border and solubilised. Dietary vitamin D_3 treatment was shown not to change the relative amounts of the 5 major core material proteins with molecular weights of 101 000 daltons; 94 000 daltons; 67 000 daltons; 42 000 daltons (actin) and 17 000 daltons. In contrast dietary vitamin D_3 treatment caused significant reductions in levels of two proteins with molecular weights of 111 000 daltons (sucrase) and 83 000 daltons, and increases in levels of a protein with molecular weight 78 000 daltons (possibly a subunit of alkaline phosphatase).

The brush border membrane proteins which show molecular weights of 111 000 daltons, 83 000 daltons, and 78 000 daltons are all readily solubilised with Triton X-100 and are located on the extracellular surface of brush-border membranes as found by labelling with ^{125}I-diazoiodosulphanilic acid and lactoperoxidase. A significant vitamin D-dependent difference was found with respect to iodination of isolated core material as shown by the ^{125}I-labelling of the 42 000 daltons protein (actin). The 42 000 dalton protein was labelled two to three times more extensively when associated with core material derived

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from vitamin D-deficient chicks. Increasing salt concentration (0 - 125mM KCl) present during core material isolation increased the probability of ^{125}I-labelling. This increase in the extent of actin iodination is coupled to a salt induced decrease in the stability of the core material which is shown by a decrease in the percentage of total brush border membrane actin which is Triton - insoluble.

This suggests that the vitamin D-induced decrease in the accessibility of actin to iodination results from a vitamin D-dependent change in the structure of the core material. These results implicate a role of dietary vitamin D_3 in maintaining a specified composition and topology of both the brush border membrane proteins as well as its associated cytoskeletal core proteins. These proteins are possibly important for intestinal calcium transport. An effect of vitamin D on the cytoskeletal structures in general, may explain the role of vitamin D in such diverse target tissues as brain and pancreas (Mooseker and Tilney, 1974; Roth et al, 1981a,b), in which bulk, transepithelial transport of calcium does not occur.

Evidence for circadian rhythms in serum levels of vitamin D-dependent CaBP and in the activity of 25(OH)-1-hydroxylase in the chick has been documented (Miller and Norman, 1982). The chick serum CaBP concentration fluctuated with a diurnal rhythm. Levels at peak periods were 2,5-fold higher than during the nadir. Appearance of circadian rhythms in serum CaBP levels and in 1-hydroxylase activity in sexually immature male chicks under constant environmental and dietary conditions, strongly suggests that these rhythms are endogenous. Other aspects of the vitamin D endocrine system may fluctuate diurnally and further studies to detect such rhythms were proposed.

In contrast to the studies conducted to establish the tissue localisation of the CaBP a great deal of controversy concerning the subcellular localisation of the CaBP in the cells with which it is associated, occurred in recent years, but this question has now been resolved. There were two schools of thought concerning the localisation of CaBP. Earlier studies indicated that the CaBP was present in goblet cells and at the absorptive surface (Taylor and Wasserman, 1970; Lippiello and Wasserman, 1975; Piazolo et al, 1975; Taylor and McIntosh, 1977). It was also thought that CaBP was present in the cytoplasm of absorptive cells but not in goblet cells (Arnold et al, 1976; Morrissey et al, 1978). As reported independently by Jande et al, (1980), (1981); Taylor, (1981); and Roth et al, (1982a) it seems to be now agreed that the CaBP is present exclusively in the cytosol fraction of the columnar epithelial cells of the chick. Taylor, (1981), showed that in his previous studies (Taylor and Wasserman, 1970) that both extracellular and goblet cell localisation of CaBP were the result of an artifactual redistribution of the CaBP. This phenomenon occurred during the manipulation associated with the thin frozen sections in the preparation for histological examination and evaluation.

It was reported that CaBP in the chick cerebellum was present in the Purkinje cells associated with the cortex (Jande et al, 1981). A complete histochemical mapping of the CaBP in the chick central nervous system has been carried out (Roth et al, 1981b). CaBP was present in the perikarya, dendrites and axons of a specific population of neurons in all areas of the CNS (Roth et al, 1981a). CaBP and positive and negative neurons of the same type were intermingled. Thus, in the chick CNS, the CaBP is a neural marker.

As a consequence of the presence of the steroid hormone complex in the nucleus of the cell, it has been shown that there is a stimulation of RNA synthesis (Tsai and Norman, 1973; Siebert et al, 1979) as well as template activity (Zerwekh et al, 1976). Following the administration of vitamin D_3, a clear stimulation of a mRNA coding for a vitamin D induced CaBP was shown (Emtage et al, 1973, 1974a, b; Spencer et al, 1976, 1978; Christakos and Norman, 1980). The amount of this mRNA for intestinal CaBP was shown to be proportional to the amount of $1,25(OH)_2D_3$ localised in the intestinal mucosa. The amount of CaBP has been shown to be correlated with the specific activity of the renal 25(OH)D-1-hydoxylase by Morrissey and Wasserman, (1971),(1977) as well as with vitamin D-mediated intestinal calcium transport.

Further studies on the molecular biology of the Chick CaBP have been carried out. Siebert et al, (1982) completed important studies to elucidate the mechanism(s) involved in the induction of the vitamin D-dependent intestinal CaBP by $1,25(OH)_2D_3$. They performed their study by examining the cell-free translation of CaBP-mRNA activity in total cellular RNA extracts of polysomes obtained from chick small intestine. The CaBP-mRNA was measured by immunochemical analysis of polypeptides synthesised upon the addition of purified intestinal RNA or polysomes to an intestinal cell lysate system. Intestinal polysomes isolated from vitamin D-deficient chicks treated with vitamin D_3, $1,25(OH)_2D_3$ or 1α-hydroxy vitamin D_3 were found to complete the synthesis of an immunoprecipitated protein which migrated with authentic chick intestinal CaBP on SDS polyacrylamide gels. A detectable level of polysomal CaBP-mRNA activity was present within 3 hours and a maximal activity achieved at 9 hours after administration of $1,25(OH)_2D_3$. Increases in the levels of cytoplasmic CaBP were first detected at 6

hours which was significantly later than the polysomal CaBP-mRNA activity. CaBP-mRNA activity in total cellular RNA, isolated with guanidine thiocyanate, was also found to be dependent on vitamin D_3 or $1,25(OH)_2D_3$ administration.

Surprisingly, total RNA and poly(A) - RNA were found to direct the synthesis and release of two additional polypeptides that were precipitated even [illegible] affinity purified antibody to CaBP. An 11 000 dalton polyp[illegible]early vitamin D-dependent whereas the other 38 000 dalton p[illegible]as not. A time course for the appearance of the CaBP-mRNA [illegible] in total cell mRNA extracts following $1,25(OH)_2D_3$ treatment was found to be similar to that observed in polysomal preparations. These results indicated that activation of CaBP biosynthesis results at least in part from $1,25(OH)_2D_3$-induced increases in levels of total cellular CaBP-mRNA activity and were therefore consistent with transcriptional regulation of CaBP biosynthesis by $1,25(OH)_2D_3$.

A recombinant cDNA library was constructed, to facilitate the study of genetic actions of vitamin D_3 and its hormonally active metabolite $1,25(OH)_2D_3$ in the initiation of the de novo biosynthesis of a 28 000 dalton vitamin D-dependent CaBP present in the chick intestine (Hunziker et al, 1983). The recombinant plasmids were prepared by the homopolymeric tailing and hybridisation method using as a starting template poly(A) - enriched mRNA obtained from the intestinal mucosa of vitamin D_3 replete chicks. Screening of the 9516 clones was effected by using a comparative in situ colony hybridisation technique with two [^{32}P]-cDNA probes. The authors identified 26 clones that consistently displayed a significantly increased hybridisation signal when comparing

the D-deficient versus the D-replete enriched probe. Further evaluation of these clones by hybrid-selected translation showed the presence of CaBP specific sequences. By RNA gel analysis of poly(A) - RNA, three independent mRNA species were found to hybridise to a CaBP clone. None of these RNA species was found to be present in the vitamin D-deficient poly(A) - RNA. With this comparative colony hybridisation procedure, the authors were able to identify CaBP specific clones corresponding to a mRNA that is 0,1% of the tota oly(A) - mRNA. The cloning of the cDNA complementary to chick intestinal CaBP will allow researchers to study the mechanism by which $1,25(OH)_2D_3$ receptors (Hunziker et al, 1982) function in stimulating CaBP genes, and also intestinal calcium transport (Tsai and Norman 1973).

The cloning of the cDNA complementary to chick intestinal CaBP could allow one to study the genomic organisation and the ancestral relationships for several vitamin D-dependent CaBPs including the 28 000 dalton chick intestinal Wasserman and Taylor, (1966) and renal CaBP Chistakos et al, (1979), the 10 000 dalton rat intestinal CaBP (Marche et al, 1977; Fullmer and Wasserman, 1981), the 34 000 dalton chick bone CaBP (Chistakos and Norman, 1978), and 9-11 500 dalton rat skin CaBPs (Laouari et al, 1980). This statement can be justified as the [^{32}P] probes used, were complementary to the appropriate DNA's.

1.4 Mammalian CaBPs

The mammalian CaBPs are by far the most studied of the vitamin D-dependent CaBPs. Proteins from the mammals have been isolated from many species including the rat, mouse, dog, guinea pig, hamster, cow, pig, horse, monkey, baboon and man. The rat has been th ost widely

studied of the mammals and CaBPs have been isolated from seven tissues in this species.

As early as 1967, the existence of a vitamin D-induced calcium binding factor was reported in the rat intestinal mucosa (Kallfelz et al, 1967). Gel chromatography indicated it was a macromolecule, presumably a protein. Researchers then intensively studied the rat intestinal protein and subsequently a vitamin D-dependent CaBP with molecular weight of 13 000 daltons was purified (Schachter, 1970). Ooizumi et al (1970) reported the existence of two vitamin D-dependent CaBPs in the rat intestinal mucosa. The molecular weights of these proteins were 24 000 and 145 000 daltons. The latter molecular weight reported, has since been discounted by many researchers, as one of the filtration methods used in the isolation of the protein, used an ultrafiltration membrane with a large pore size. This excluded all lower molecular weight proteins from being retained and characterised.

Another study was conducted by Drescher and DeLuca, (1971) in which they isolated an intestinal vitamin D-dependent CaBP of molecular weight between 8-9 000 daltons as shown by sedimentation equilibrium ultra centrifugation. They purified a similar protein of molecular weight 13 000 daltons using gel filtration and electrophoresis which could have been a precursor for the smaller protein. A vitamin D-dependent CaBP from rat intestinal mucosa with a molecular weight of 12 000 daltons was isolated by another group (Harrison et al, 1975). Freund and Bronner (1975) found by analytical gel electrophoresis two vitamin D-dependent CaBPs with similar molecular weights. Moriuchi et al (1975) also demonstrated two different vitamin D-dependent intestinal CaBPs in the rat. The larger protein was found predominantly in the jejunum and ileum

and had a molecular weight of 27 000 daltons and bound 6 nmoles Ca/mg of protein. The smaller protein was associated mainly with the duodenum and jejunum and had a molecular weight of 12 500 daltons and demonstrated high affinity for calcium.

Miller et al, (1979) isolated a vitamin D-dependent CaBP from brush borders of rat duodenal mucosa. The molecular weight of the protein was 19 000 daltons against the isolated form of cytosolic duodenal CaBPs namely 11 000 daltons. The lower molecular weight protein has been shown to be more vitamin D-dependent and have a greater affinity for calcium. Kowarski and Shachter (1980) investigated a vitamin D-dependent activity in the particulate fractions of rat mucosal homogenates. From this study they aimed to confirm the involvement of this activity in calcium transport, to solubilise it, and to isolate the responsible intrinsic membrane protein as a component of the cation transport system. They isolated a new intestinal membrane CaBP which they named IMCal. IMCal has an approximate molecular weight of 200 000 daltons. When this protein was dissociated in 2% SDS it revealed on electrophoresis a monomer corresponding to a molecular weight of 20 450 daltons. It was noted that the monomer was clearly distinct from the soluble rat CaBP which migrates more rapidly in SDS gels corresponding to a molecular weight of 11 500 daltons. The 200 000 dalton protein was shown to have a mean pk of 6,4. This IMCal protein on amino acid analysis, was shown to contain a relatively high content of arginine, aspartic acid, histidine, leucine and tyrosine whereas rat CaBP contains relatively more glutamic acid. Gleason and Lankford, (1981), purified a rat intestinal CaBP achieving a final purification on ion-exchange chromatography. The protein was found to be heat stable and had a molecular weight of 11 500 daltons. The protein also showed electrophoretic behaviour characteristic of rat intestinal CaBPs purified by other techniques.

As has thus far been shown, there is some variation in the molecular weights reported for the intestinal forms of one mammalian species. Most groups reported results from 10-13 000 daltons. These variations could in part have been attributed to the varying isolation procedures used to purify the protein as well as limited proteolysis of the protein. It should be noted that no immunochemical comparisons have been drawn between the various molecular weight CaBPs isolated.

Other than the intestinal forms of CaBP studied and characterised, further studies on tissue distribution in the rat have been described. A vitamin D-dependent CaBP with molecular weight 28 000 daltons was purified from the rat kidney (Roth et al, 1981a). Studies on the rat placenta revealed a CaBP closely related to that of the intestinal form with a molecular weight of 10 500 daltons (Bruns et al, 1978). The vitamin D-dependency of the salivary gland CaBP from the rat submandibulary gland was shown (Freund et al, 1977). The parotid glands were shown to contain more CaBP activity than the submandibulary glands and were shown to be vitamin D-dependent whereas the latter protein showed no vitamin D-dependency. Two vitamin D-dependent CaBPs were isolated in the rat skin (Laouari et al, 1980). It was shown that the major 11 500 dalton CaBP was situated closest to the basal layer of the epidermis. (Saurat et al, 1981; Pavlovitch et al, 1983). This rat skin CaBP has the same molecular weight and similar composition as skeletal muscle parvalbumin as shown by partial amino acid analysis, which did show small differences in sequences 75-99 (Rinaldi et al, 1982). MacManus et al, (1985) has just shown that only one major low molecular weight CaBP could be isolated by HPLC procedures from aqueous extracts of homegenised adult rat skin. This was shown by tryptic peptide mapping and independent amino acid sequence analysis to be identical in

all 109 residues with the parvalbumin from rat skeletal muscle. The CaBP was not found in the skin epidermis, but was confined to the dermal layer. The rat skin CaBP is therefore now shown to be parvalbumin.

Bruns et al, (1981) purified an intestinal and placental CaBP from the mouse with similar molecular weights. The mouse intestinal protein had a molecular weight of 10 000 daltons and demonstrated immunological identiy to the rat intestinal CaBP. The placental CaBP was shown also to have a molecular weight of 10 000 daltons on SDS polyacrylamide gels. Biochemical evidence was presented (Delorme et al, 1983) for the presence of two vitamin D-dependent CaBPs in the mouse kidney. These proteins were very different biochemically and exhibited no cross-immunoreactivity. The one had a molecular weight of 25 000 daltons and the other 10 000 daltons. The latter protein was immunologically identical with the mouse duodenal 10 000 dalton CaBP. Moreover, the kidney contained about twice as much 10 000 dalton CaBP as 25 000 dalton CaBP. The mouse 10 000 dalton renal CaBP was already reported to be present during foetal life and reached its adult level during the first week after birth. The concomitant presence of substantial amounts of the two vitamin D-dependent CaBPs in the mouse kidney is peculiar to the organ of this animal, and might consequently provide a unique model for studying at the genetic level the hormonal expression of $1,25(OH)_2D_3$.

Alpers et al, (1980) isolated two vitamin D-dependent CaBPs from the intestinal mucosa of the dog. The duodenal and upper jejunal CaBPs were shown to have a molecular weight of 19 000 daltons. The ileal CaBP was shown to have a molecular weight of 57 000 daltons. It was suggested that the small molecular weight CaBP could be derived at least in part from the larger protein by proteolysis but that the small protein was

relatively resistant to trypsin. The demonstration of CaBP activity with a large molecular weight in dog ideal mucosa provides further evidence for the role the ileum might play in regulating calcium transport and absorption.

Studies on the intestinal as well as the parathyroid CaBP in the dog were carried out to elucidate the biochemical nature of these proteins (Oldham et al, 1980). The two proteins reported by these authors responded differently to changes in vitamin D-status. The intestinal-protein was shown to be vitamin D-dependent and the parathyroid protein was not. The physiological significance of this observation has yet to be shown.

It was shown by Fullmer and Wasserman, (1975) that the molecular weight of the guinea pig CaBP was 11 000 daltons as determined by gel filtration chromatography and 8 900 daltons by amino acid analysis. In further studies conducted on young adult guinea pigs, Chapman et al, (1977) detected and reported the distribution of an intestinal CaBP. They found a CaBP that eluted off G-100 Sephadex gel filtration with a low molecular weight.

The Golden hamster has also been studied and a CaBP was isolated from the intestinal mucosa (Kallfelz and Wasserman, 1972). They showed a correlation between ^{45}Ca absorption and intestinal calcium binding activity. The authors also reported that in the hamster unlike the rat, calcium transport is greater in the ideal tissue than in the duodenal gut sacs.

The bovine intestinal CaBP was isolated and purified by Fullmer and Wasserman, (1975). They showed the CaBP to have a molecular weight of 11 000 daltons by gel filtration and 9 000 daltons by amino acid analysis. No species cross-reactivity was shown when tested against antisera prepared in response to the chick intestinal CaBP. The crystal structure of this protein has been determined recently (Szebenyi et al, 1981).

The pig is yet another of the numerous mammalian species studied. Hitchman and Harrison, (1972) purified a CaBP in duodenal mucosa of the pig. They showed that the pig contained a CaBP with a similar molecular weight reported for the rat (12 - 13 000 daltons), but dissimilar to the molecuar weight of the protein from the chick. In addition to this the pig intestinal mucosa contained a higher molecular weight protein which was probably not vitamin D-dependent. The same authors purified a porcine vitamin D-induced intestinal CaBP, but did not determine its molecular weight (Hitchman et al, 1973). Bryant and Andrews, (1983) isolated a vitamin D-dependent CaBP from duodenal mucosa of the pig and other mammals (sheep and rabbit). The molecular weights of the three proteins were determined and shown to be approximately 8 800 daltons. A pig intestinal CaBP was found to have a molecular weight of 9 000 daltons by amino acid analysis and 11 000 daltons by Sephadex gel filtration chromatography described (Fullmer and Wasserman, 1975).

Oldham et al, (1974) isolated a CaBP from normal porcine parathyroid glands. The molecular weight was shown to be 15 000 daltons by calibrated gel filtration. A molecular weight estimate of 11 700 daltons was obtained from sucrose density gradient centrifugation. In addition to the tissue distribution and vitamin D-dependency of non-primate species, studies have also been conducted on higher mammals including the

monkey, baboon and man.

Evidence for a vitamin D_3-induced CaBP in new world primates namely the squirrel and cebus monkeys was described by Wasserman and Taylor, (1971). Administration of vitamin D_3 increased the relative calcium binding activity of the duodenum and jejunum in both species. Little or no difference was noted in the calcium binding activity of the material from the ileum. The comparative binding of calcium from the different segments followed the sequence duodenum > jejunum > ileum, a result similar to that observed in the chick (Taylor and Wasserman, 1967). Acrylamide gel patterns showed the presence of a protein staining band which was present in the supernatant fluid of vitamin D-replete monkeys but absent in D-deficient supernatant fluid. The vitamin D-dependent band in the squirrel monkey intestinal supernatant fluid migrated slightly slower than the chick CaBP. This result suggests that the squirrel monkey CaBP has a lower charge density and/or a greater molecular size. These data indicated that the intestinal mucosa of primates, like that of chick, rat and dog is capable of synthesising a calcium binding factor in response to vitamin D_3.

An intestinal CaBP protein from baboon was isolated by Gleason and Lankford, (1982) and was claimed to have similar electrophoretic behaviour to non-primate forms excluding the rat. These authors did not determine the vitamin D-dependency of the protein nor did they determine the molecular weight. These authors showed that an important difference exists between the rat intestinal CaBP and the protein isolated from the baboon intestine. Studies indicated that the rat CaBP shows relatively less anodic mobility and is therefore less negatively charged than the baboon protein. The baboon CaBP showed rapid anodic mobility on SDS gels

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