ISOLATION OF A VITAMIN D-DEPENDENT, CALCIUM-BINDING PROTEIN FROM BRUSH BORDERS OF RAT DUODENAL MUCOSA

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1. Introduction

The vitamin D-dependent calcium-binding protein found in the mammalian duodenum is a small molecular weight (9000–12 000), fairly acidic protein with 2 calcium-binding sites [1,2]. CaBP appears to be a cytosolic protein [3–5], but has also been detected in the plasma membrane [6,7]. It is generally accepted that the brush border membrane is a major site of the cellular action of 1,25-dihydroxyvitamin D_3 and that duodenal CaBP is the best-known molecular expression of the hormone-like action of vitamin D [8]. However, CaBP has not been detected in the brush borders of chick intestinal epithelium [1], nor has it been reported in rat brush borders. A calcium-dependent adenosine triphosphatase [9] and a particulate calcium-binding complex of high molecular weight [10] that are stimulated by vitamin D have been identified in rat intestinal brush border preparations.

This report describes the isolation and some characteristics of a new vitamin D-dependent, calcium-binding protein from purified brush borders of rat duodenal mucosa.

2. Experimental procedures

2.1. *Animals and diets*

Male Sprague Dawley rats, purchased from ARS, Madison, WI were fed a low calcium (0.06%) and low phosphorus (0.2%) semisynthetic regimen, diet I [11], for several days prior to sacrifice.

To study the effect of vitamin D deficiency, weanling animals (40–50 g) were divided into two lots, fed either diet I (2200 IU vitamin D_2/kg) or the same diet without added vitamin D_2 (I–D). After ~ 7 weeks, half of the I–D animals were injected intraperitoneally with 20 IU (≃ 0.5 μg) of 1,25-$(OH)_2$-D_3, 14–16 h prior to sacrifice. The control I–D animals received injections of vehicle. Vitamin deficiency was assessed by measuring plasma calcium and duodenal CaBP levels as in [5,12].

2.2. *Isolation of purified brush borders*

Animals (125–250 g) were killed by cervical dislocation. Sections of the duodena (~ 10 cm) were excised and placed in cold 0.9% NaCl, slit longitudinally, washed free of intestinal content with saline solution and the mucosae were collected by scraping with a glass slide. The mucosal scrapings were suspended in 75 ml 5 mM Na_3-EDTA plus 1 mM Hepes–Tris buffer (pH 7.5) and homogenized (12 strokes/min) with a motor-driven glass teflon homogenizer at a setting of 50–60 on the Powerstat variable transformer. Purified brush borders were isolated from the homogenate by differential centrifugation [13]. All operations were carried out at 0–4°C.

2.3. *Preparation of brush border S-100*

Purified brush borders were homogenized in Tris-buffer [14] and the resulting homogenate was centrifuged for 1 h at 100 000 × *g*. The supernatant,

Abbreviations: CaBP, vitamin D-dependent calcium-binding protein from duodenal cytosol; 1,25-$(OH)_2$-D_3, 1,25-dihydroxyvitamin D_3; Na_3-EDTA, trisodium ethylenediaminetetraacetic acid; Hepes, *N*,2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, Tris (hydroxymethyl) aminomethane

designated S-100, was dialyzed (24 h) against 0.03 mM phenylmethyl sulfonyl fluoride in 0.02 M ammonium acetate, pH 7.2 at 4°C. The dialyzate was lyophilized and either used immediately or stored at −15°C.

2.4. *Sephadex gel filtration*

The S-100 fraction was dissolved in ammonium acetate buffer, centrifuged at 1500 rev./min for 5 min and the supernatant applied to a Sephadex G-50 or G-100 column equilibrated with buffer containing 7 μM $CaCl_2$ plus $^{45}CaCl_2$ (New England Nuclear, Boston, MA). Unless stated otherwise, the calcium-binding elution profile was obtained by using a descending flow rate of 6 ml/h and the fractions were assayed for radioactivity in Bray's scintillation fluid [15] with a Nuclear Chicago (Unilux IIA) liquid scintillation spectrometer. Calcium binding was determined by a modification [12] of the method in [16] and binding activity was expressed as nmol Ca^{2+}_{bound}/mg protein loaded on the column. Protein concentrations were determined spectrophotometrically [17] or according to [18] with crystalline bovine serum albumin as the standard.

2.5. *Polyacrylamide disc gel electrophoresis*

The detailed methods used for electrophoresis have been described [19]. Briefly, the separating and stacking gels contained 7% and 2.5% polyacrylamide, respectively, and the running buffer in the electrode chamber was Tris–glycine (pH 8.3). The calcium concentration of the anode chamber buffer was 1 μM, spec. radioact. ~ 10 μCi/μmol. To determine calcium-binding activity, gel slices (1 mm) were solubilized overnight in 0.5 ml 30% H_2O_2 at 55°C and assayed for radioactivity in Aquasol (New England Nuclear, Boston, MA). Protein staining patterns of duplicate gels were obtained as in [19].

3. Results

The elution profile obtained by ascending chromatography on Sephadex G-50 for pooled S-100 samples of duodenal brush borders from rats on diet I is shown in fig.1. Peak B, $v_e/v_o = 1.22$, which differed in location compared to cytosolic CaBP, $v_e/v_o \simeq 1.4$ [19], had a binding activity of 0.14 nmol Ca^{2+}_{bound}/mg protein loaded. Appropriate fractions of peak B were combined and further subjected to descending chromatography on Sephadex G-100. The elution profile of partially-purified peak B material (insert fig.1) indicated a binding activity of 7.0 nmol Ca^{2+}_{bound}/mg protein, a purification of ~ 50-fold.

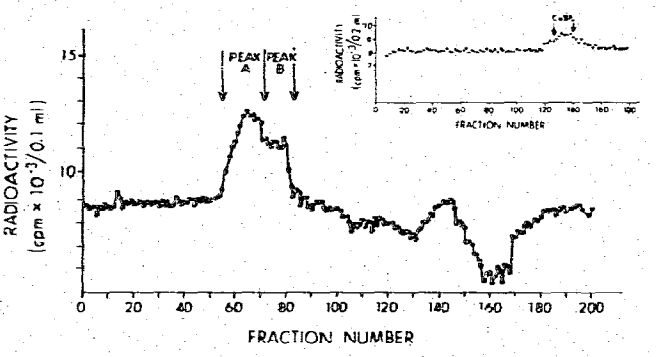

Fig.1. Elution profile of brush border supernatant. S-100 samples from purified brush borders of animals on diet I were pooled and 15.3 mg protein were applied to a Sephadex G-50 (fine) column (1.5 × 80 cm). Separation was effected by ascending chromatography. The flow rate was 10 ml/h and 0.1 ml aliquots of 1 ml fractions were assayed for radioactivity. Peak A designates the void volume and peak B ($v_e/v_o = 1.22$) the brush border calcium-binding protein. The insert shows the elution profile of peak B material rechromatographed on Sephadex G-100. Protein (0.35 mg) was applied to a column, 0.65 × 100 cm. Separation was effected by descending chromatography at a flow rate of 6 ml/h. Fractions (0.2 ml) were collected and assayed for radioactivity.

The molecular weight of brush border calcium-binding protein, as estimated from a Sephadex G-100 column calibrated with blue dextran, cytochrome *c*, myoglobin, α-chymotrypsinogen-A, and pepsin was 18 500 ± 3000. This value is considerably larger than that determined for the cytosolic duodenal CaBP, mol. wt 11 000 [5]. The electrophoretogram and gel tracings of partially-purified brush border calcium-binding protein illustrated in fig.2 substantiate the presence of a major calcium-binding and two minor, barely discernible protein bands. The R_F values determined for the major band and cytosolic CaBP were 0.77 and 0.17, respectively. Cytosolic CaBP was not detected in the brush border S-100 preparations (fig.2).

The effects of vitamin D deficiency and the administration of 1,25-$(OH)_2$-D_3 on brush border

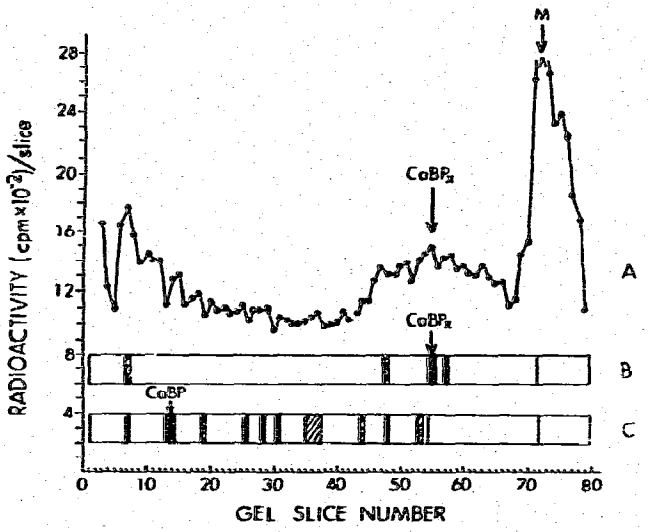

Fig.2. Binding activity profile (A) and protein staining pattern (B) of partially purified brush border protein subjected to polyacrylamide disc gel electrophoresis. A protein staining pattern of the S-100 from duodenal mucosa of 23 day old rats is shown for comparison (C). CaBP refers to cytosolic, vitamin D-dependent, calcium-binding protein (mol. wt 11 000); CaBP$_x$ refers to the brush border calcium-binding protein and M to bromphenol blue marker.

calcium-binding protein levels are shown in fig.3. No calcium-binding activity was detectable in the peak B region of the brush border preparation from vitamin D-deficient animals (fig.3A). Intraperitoneal administration of 0.5 μg 1,25-$(OH)_2$-D_3 to vitamin D-deficient littermates 16 h before sacrifice resulted in a marked increase in binding activity, to ~ 1.25 nmol Ca^{2+}_{bound}/mg protein (fig.3B). In a second experiment, the binding activity of the 1,25-$(OH)_2$-D_3-induced brush border protein was 1.92 nmol Ca^{2+}_{bound}/mg protein, whereas that in material isolated from vitamin D-replete animals was 2.45 nmol Ca^{2+}_{bound}/mg protein.

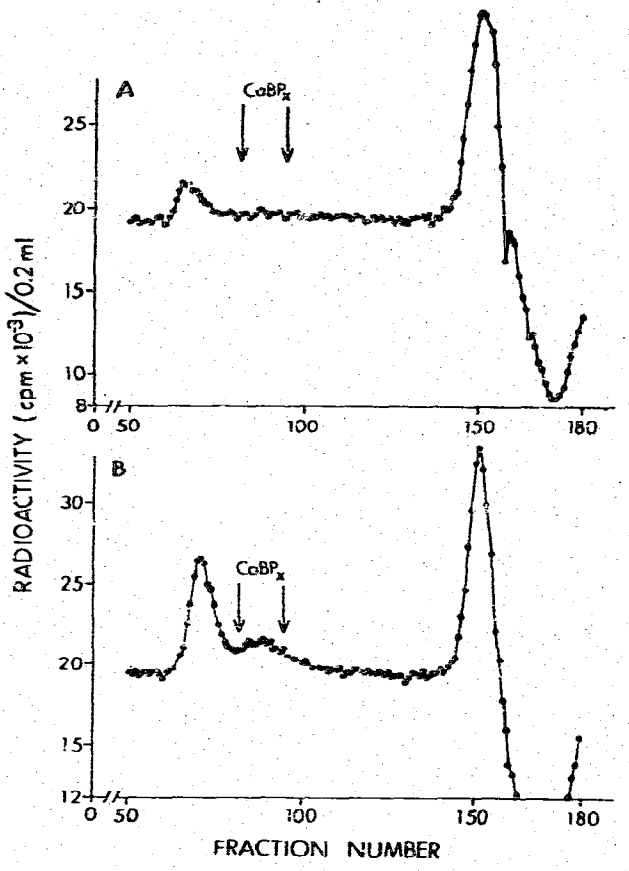

Fig.3. Elution profiles of dialyzed brush border S-100 from vitamin D-deficient animals (A) and vitamin D-deficient animals treated with 20 IU (≃ 0.5 μg) 1,25-$(OH)_2$-D_3 16 h prior to sacrifice (B). Protein, 1.65 mg for the untreated and 1.50 mg for the treated group, was applied to a Sephadex G-100 column (0.65 × 100 cm). Separation was effected by descending chromatography; the flow rate was 6 ml/h and 0.2 ml fractions were assayed for radioactivity.

4. Discussion

Two soluble vitamin D-dependent calcium-binding proteins in rat small intestine have been identified [20,21]. One, found predominantly in the jejunum and ileum, had mol. wt 27 000; the other, associated primarily with the duodenum, had mol. wt 12 500. The latter protein was more vitamin D-dependent, had a greater affinity for calcium, and appears identical with the cytosolic duodenal CaBP. The ileal calcium-binding protein differs from the brush border binding protein isolated here in intestinal location, molecular size and mode of isolation. Vitamin D-dependent calcium-binding activity of relatively high molecular weight, 0.5 × 10^6, has been isolated from the particulate fraction of rat intestinal brush borders [10]. In addition, a calcium-dependent ATPase was observed [9] in the brush border of rat small intestine which was increased markedly after administration of vitamin D to vitamin D-deficient animals. The app. mol. wt. 18 500, of the brush border calcium-binding protein isolated in this

study differentiates it from cytosolic duodenal CaBP (mol. wt 11 000), as well as from the larger particulate species [9,10].

Partially purified brush border calcium-binding protein (insert, fig.1) binds ~ 350 nmol Ca^{2+}/mg protein, a binding activity comparable to that of cytosolic CaBP, 200 nmol Ca^{2+}_{bound}/mg protein [5].

Since the translocation of calcium across the brush border membrane has been shown to involve considerable binding to membrane components as well as transport into the intravesicular space (A.M., Reid, F.B., in preparation) it seems reasonable to assume that cation movement may be associated with a carrier or calcium-binding protein. Whether brush border calcium-binding protein is directly involved in calcium transport remains to be established. The presence of a calcium-binding protein in the intestinal brush border, its increase in response to 1,25-$(OH)_2$-D_3, and the decreased calcium uptake by brush border membrane vesicles from vitamin D-deficient rats (A.M., Reid, F.B., in preparation) suggest a functional role in the uptake process.

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