PRIMARY TRANSLATION PRODUCT OF mRNA CODING FOR RAT DUODENAL VITAMIN D-DEPENDENT CALCIUM-BINDING PROTEIN

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1. Introduction

The intestinal mucosa of several species contains vitamin D-dependent calcium-binding proteins (CaBP) which may be involved in the complex process of calcium transport [1]. The cytosolic CaBP (11 500 Mr) [2,3] of the rat duodenum accounts for 2% of the soluble mucosal protein [4]. It also exhibits considerable specificity since it has no immuno-crossreactivity with either other mammalian intestinal CaBPs [5], including porcine CaBP [5-7], or the larger (28 000 Mr) [8] vitamin D-dependent CaBP of the chick duodenal mucosa [5].

The induction of intestinal 28 000 Mr CaBP synthesis by active messenger RNA (mRNA) has been studied in the chick [9-12]. It appears to be dependent on the hormonal metabolite of vitamin D, 1,25-dihydroxycholecalciferol (1,25-(OH)2D3) [11]. In the rat there is evidence indicating that the 11 500 Mr duodenal CaBP concentration measured by using radioimmunoassay is dependent upon 1,25-(OH)2D3 [13,14]. To date, however, there is no report of its de novo synthesis.

To further study the regulation of rat duodenal CaBP production we have undertaken an analysis of the expression of the relevant gene(s). This report deals with the identification of the primary translation product of the mRNA coding for rat duodenal CaBP.

2. Materials and methods

2.1. Animals

Five-week-old male Sprague-Dawley rats (Charles River, France) maintained on a commercial complete rat diet (UAR 113, 0.8% Ca2+; 0.7% P; 2000 IU vitamin D3/kg) were used.

2.2. Isolation and purification of rat intestinal calcium-binding protein

Calcium-binding protein was isolated and purified by successive gel filtration and anion exchange chromatography [3]. The purified CaBP migrated as a single band on SDS-polyacrylamide gel electrophoresis. Its binding capacity for calcium assayed by a competitive binding assay [15] was ~200 nmol Ca2+ bound/mg protein. The purified CaBP obtained in this way was used to prepare both rabbit antiserum and iodinated CaBP as in [7].

2.3. Total cellular RNA extraction

The animals were exsanguinated. The duodenum was isolated, removed (free of pancreatic fragments), everted and washed in cold, sterile 0.9% NaCl. The mucosa was collected with a glass slide at 4°C, weighed and immediately frozen in liquid nitrogen. Total RNA was isolated from the tissue according to [16] using phenol/chloroform/isooamyl alcohol (50/50/2) extractions and successive ethanol and LiCl precipitations.

2.4. Purification of poly(A+)-RNA

Poly(A+)—RNA was separated by affinity chromatography on oligo (dT)-cellulose T3 (Collaborative Res.) and collected after ethanol precipitation [17]. Poly(A+)—RNA was then dissolved in sterile H2O at 1 µg/ml and stored at −70°C.

2.5. Cell-free translations

Translations of RNA (0.5–1.0 µg) were carried out in 10 µl rabbit reticulocyte lysate (Amersham) containing 20 µCi L-[35S]methionine (NEN) in the conditions recommended by the supplier of the lysate. Translations were performed at 30°C and for
60 min. After incubation, 1 µl aliquots were removed and spotted on Whatman 3 MM disks. Proteins were precipitated by 20% trichloroacetic acid. The disks were boiled for 10 min in 1 liter of 10% trichloroacetic acid and washed successively with cold 10% trichloroacetic acid, ethanol, ethanol–ether (1/1) and ether [18]. Radioactivity was counted in 10 ml liquid scintillator.

2.6. **Specific immunoprecipitations**

Aliquots (25 µl) of the labelled cell-free translation products were diluted 1:5 with sodium phosphate buffer 0.1 M (pH 7.4) containing 0.1% bovine albumin, 0.1% methionine, 0.02% sodium azide and 1% Triton X-100. Samples were incubated overnight at 4°C with 1 µl undiluted rabbit antiserum prepared against purified rat duodenal CaBP [7] or with 1 µl non-immune serum. To test for the specificity of antibody binding, several samples were treated with 10 µg unlabelled purified CaBP before addition of antisera. Goat antiserum (50 µl) to rabbit γ-globulin (Miles) were added to each immunoprecipitation reaction and the samples were incubated at 4°C for an additional 24 h in 1.5 ml propylene tubes (Eppendorf). Incubation mixtures were laid on 300 µl 1 M sucrose solution in the incubation buffer. Immunoprecipitates were collected through the sucrose-cushion by 5 min centrifugation in an Eppendorf microfuge and washed 6 times with 1 ml immunoprecipitation buffer.

2.7. **Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)**

Translation products and immunoprecipitates were analyzed by electrophoresis on 12% or 15% acrylamide as a running gel and 5% stacking gel [19]. Immunoprecipitates were dissolved in 20 µl sample buffer (60 mM Tris (pH 6.8), containing 2% SDS and 5% β-mercaptoethanol) incubated 1 h at 37°C. All samples were then incubated at 100°C for 3 min in 20 µl sample buffer. 14C-Labelled proteins of known Mr (NEN), as well as purified 125I-labelled (125I-) CaBP and immunoprecipitated 125I-CaBP, served as markers in adjacent gel lanes. Using a Kodak intensifying screen for periods of 1–7 days, autoradiograms were prepared by exposing the vacuum-dried gels to Kodak X-O-Mat AR film at −80°C.

3. **Results**

The results of isolation, purification, translation of mRNA in the reticulocyte lysate cell-free system and immunoprecipitation of the translated products are presented in table 1. The yield of mRNA after

<table>
<thead>
<tr>
<th>Mucosa weight (g)</th>
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<tr>
<td>Total nucleic acids (mg)</td>
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<td>Total RNA (mg)</td>
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<tr>
<td>Poly(A⁺)– RNA (µg)</td>
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<tr>
<td>A₂₆₀/A₂₅₀</td>
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<td>A₂₆₀/A₂₃₅</td>
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<tr>
<td>[³⁵S]Methionine incorp./µg poly(A⁺) (cpm X 10⁻³)</td>
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</tr>
<tr>
<td>[³⁵S]Methionine immunoprecip./µg poly(A⁺) (cpm X 10⁻³)</td>
<td>31</td>
</tr>
<tr>
<td>% Immunoprecipitated</td>
<td>28</td>
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Fig.1. Analysis of total products of cell-free translations (rabbit reticulocyte lysate) of mRNAs prepared from rat duodenum. ³⁵S-Labelled proteins in the translation mixture were analyzed by electrophoresis on 0.1% SDS 15% polyacrylamide gel slabs and visualized by autoradiography: (A) ¹⁴C calibration proteins, downward arrow denotes ovalbumin 46 000 Mr; carbonic anhydrase 30 000 Mr; lactoglobulin 18 367 Mr; cytochrome c 12 300 Mr; (B) pure ¹²⁵I-CaBP; (C) total translation products directed by duodenal mRNAs.
2 cycles of affinity chromatography on oligo (dT)-cellulose was ~1% of the total extracted RNA. In the rabbit reticulocyte lysate system, this fractionated mRNA stimulated incorporation of labelled methionine into trichloroacetic acid-precipitable protein 6-25-fold over the endogenous reticulocyte lysate protein synthesis without added mRNA. Maximum incorporation was achieved with 1 μg rat duodenal mRNA in 10 μl reticulocyte lysate. Specific immuno-precipitable [35S]methionine-labelled translated products accounted for 28% of the total protein synthesized (table 1).

SDS–PAGE analysis of the total translation products (fig.1C) revealed the presence of 3 major bands (1 with an Mr of 46 000 and 2 in the region of pure 125I-CaBP) in addition to several minor components. This suggests that the mRNA was not cleaved since high Mr [35S]proteins were synthesized. Moreover when translation was performed with 0.5 μg virus RNA (turnip yellow mosaic virus) [18] we observed protein bands of Mr ~ 150 000 (not shown). The multiplicity of translation products derived from intestinal mRNA also reflects the difficulties inherent in attempting to identify a specifically labelled duo-

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Fig.2. Analysis of immunoprecipitated products of cell-free translations (rabbit reticulocyte lysate) of mRNAs prepared from rat duodenum. Details of immunoprecipitations are described in section 2. 35S-Labelled proteins in immunoprecipitates prepared from the translation mixtures were analysed by electrophoresis on 0.1% SDS–12% polyacrylamide gel slabs and visualized by autoradiography: (A) 125I-CaBP immunoprecipitated with the double antibody system of rabbit anti-CaBP antiserum and goat anti rabbit IgG immunoglobulin; (B) 14C calibration proteins as in fig.1A; (C) immunoprecipitate of the total translated products shown in fig.1C with the double antibody system; (D) as (C) but incubation in the presence of 10 μg authentic purified duodenal CaBP; (E) as (C) but no exogenous mRNA added to the translation reaction; (F) pure 125I-CaBP.
denal molecule in studies of protein biosynthesis in intact intestinal tissues. However, when cell-free translation products derived from duodenal mRNA were treated with antiserum to CaBP, only one major protein was immunoprecipitated (fig.2C). This intestinal CaBP-related translation product was electrophoretically indistinguishable from authentic, purified 125I-CaBP (fig.2F) as well as from the immunoprecipitated 125I-CaBP (fig.2A). The addition of 10 µg unlabelled purified duodenal CaBP to the translation products prevented the immunoprecipitation of the Mr 11 500 protein (fig.2D) which was not seen in the absence of exogenous mRNA (fig.2E), attesting to the specificity of the immunoprecipitations.

4. Discussion

The demonstration of duodenal mRNA coding for the 11 500 Mr CaBP indicates that a CaBP gene is expressed in the rat intestine. Although a larger 28 000 Mr CaBP has been studied in the chick [9–12], no studies have been reported of the de novo biosynthesis of CaBP in rat intestinal tissue.

The only other study of the in vitro translation of mammalian 11 500 Mr CaBP reported to date is on pig intestinal protein [20]. Although the rat and pig 11 500 Mr CaBPs are of a similar size [2,3] and are found in similar locations [21,24], there appears to be a number of interesting differences between the 2 systems. In particular, no cross-reactivity between the 2 molecules was observed [5–7] and only pig CaBP has been shown to contain no methionine [21]. Furthermore, the translation of mRNA extracted from pig duodenal mucosa [20] revealed the presence of 2 proteins specifically immunoprecipitated by CaBP antibodies. A first band which comigrated with authentic CaBP when translation of the added messenger was carried out in the presence of leucine was considered [20] to be the translation product of CaBP mRNA. A second band had a larger molecular weight than CaBP and was present only when incorporation was carried out in the presence of labelled methionine. From [20] no evidence exists for the relationship between the two bands. We have not observed such a specific immunoprecipitable higher-Mr protein in our studies.

We detected a single translation product directed by the duodenal mRNA that is related to CaBP by the criterion of immunoprecipitation. This product not only migrated in the same position as authentic CaBP but was also displaced from immunoprecipitates by cold pure CaBP. Therefore our results suggest that either rat CaBP is synthesized, as a very similar Mr precursor containing the initiation methionine, or that rat CaBP, in contrast to pig CaBP [21], contains methionine. In the latter hypothesis one would have to conclude that CaBP synthesis does not require a larger Mr precursor. This is compatible with the intracellular cytoplasmic localization of CaBP [22], as proteins of this type require no hydrophobic leader [23]. Similar data were obtained from the larger 28 000 Mr CaBP in the chick [9,12]. Originally, de novo synthesis of a protein having similar molecular and antigenic properties to the chick intestinal CaBP was reported in [9].

Since our earlier studies have shown that rat duodenal CaBP concentrations, as well as the electrophoretic distribution of purified CaBP, depended on dietary calcium [15], phosphorus [25] and vitamin D [2], intake and 1,25-(OH)2D3 administration [4,13,14], further studies on modification of the level of mRNA coding for CaBP in these experimental conditions will provide more details on the manner in which these controls are exercised.

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References