# DETECTION OF VITAMIN D-DEPENDENT CALCIUM-BINDING PROTEIN IN THE REGION OF APICAL GLYCOCALYX OF CHICK ENTEROCYTES

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## 1. Introduction

Vitamin D is known to stimulate $Ca^{2+}$-absorption in the small intestine of animals. Many investigations [1–3] have shown that enhanced calcium absorption is closely related to biosynthesis of the specific calcium-binding protein (CaBP) in the intestinal mucosa. However, the molecular mechanism through which CaBP acts on $Ca^{2+}$-transport is not yet understood. The protein may not have a direct role in $Ca^{2+}$-transport and act only as a protector, binding the surplus of the physiologically active cation in the cell's cytoplasm [4,5].

Important information for understanding the mode of CaBP action is its cellular and subcellular localization. If the protein is localized in the cytoplasm, then, apparently, it will act as a regulator of intracellular [$Ca^{2+}$]. Although some results [5,6] confirm the presence of CaBP inside the cell and are consistent with its suggested role as a protector, a series of other investigations [7–9] convincingly show that CaBP is localized on the surface of the intestinal epithelium in the brush border region. Thus, to solve the problem of CaBP localization, new investigations and new methodological approaches are necessary.

The most used method to study the cellular localization of proteins, including CaBP, is the method of indirect immunofluorescence. Here, we report the application to the problem of CaBP localization of a new and direct method, the agar replica method [10] to study the distribution of some enzymes in the intestinal mucosa.

The agar replica method allows one to gently separate, without destroying the structure of the mucosa, the apical part of the glycocalyx, and to determine enzymes present in it.

Using this method we were able to show that a certain part of CaBP is present in the apical glycocalyx of chick enterocytes.

## 2. Materials and methods

Experiments were carried out on the duodenum of 3-week-old chicks brought up on a vitamin D-deficient diet.

The first group of chicks was given, per os, 500 IU vitamin $D_3$ in oil 72 h before sacrifice. The second group was not given vitamin $D_3$ and was used as a control.

Agar replicas were obtained according to [10]. After decapitation, the abdomen of the chick was opened, the duodenum removed and rinsed with an ice-cold solution of NaCl (0.75%). A 5 cm segment of the intestine was tied at one end and rapidly filled with 3% agar solution kept at 42°C. After filling, the intestinal segment was tied at the other end and placed in cooled saline in order to gel the agar. After 20 min the segment was taken out of the saline, cut longitudinally, and the cylinder of agar (the agar replica) separated carefully. The mucosa was scraped off from the intestinal segment and weighed as was the replica. Both were later homogenized separately in buffer solution (20%, w/v) with a homogenizer with a Teflon pestle. The composition of the buffer solution used was 0.0137 M Tris, 0.12 M NaCl and 4.74 M KCl, adjusted to pH 7.4 with HCl. The homogenate was centrifuged at 22 000 × *g* with cooling for 60 min. The supernatant fraction was assayed for total soluble protein by the biuret reaction [11] and for CaBP, using a radial immunodiffusion assay [12]. The integrity of intestinal mucosal cells after removal of the agar replica was checked by the activities of the mem-

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brane marker enzymes invertase [13] and alkaline phosphatase, with sucrose and sodium β-glycerophosphate [14] as substrates, respectively. As a cytoplasmic enzymic marker the activity of lactate dehydrogenase was determined using lithium lactate as substrate and a special reagent kit (Farb-Schnelltest für LDH; VEB Arzneimittelwerk, Dresden). Results of activity measurements are expressed as μmol . g-mucosa$^{-1}$ . min$^{-1}$ at 37°C. For alkaline phosphatase the reaction velocity is expressed as μmol $P_i$ liberated . g-mucosa$^{-1}$ . min$^{-1}$ at 37°C [15].

## 3. Results

As was to be expected, in chicks fed on a vitamin D-deficient diet no CaBP was detected either in the duodenal mucosa or in its agar replica. Results obtained on chicks given vitamin $D_3$ 72 h before the experiment are summarized in table 1. These data show that the agar replica when separated from the mucosa contains a considerable amount of CaBP; 16.1% of its total intestinal content. At the same time the portion of other soluble proteins found in the replica is essentially lower (9.5%).

Table 1
Distribution of CaBP between the duodenal mucosa and its agar replica

| Proteins | n | Agar replica[a] | | Mucosa after removing the replica | |
|---|---|---|---|---|---|
| | | Content | % Total content in mucosa[b] | Content | % Total content in mucosa[b] |
| CaBP (mg . g-mucosa$^{-1}$) | 15 | 0.76 ± 0.08 | 16.1 | 3.96 ± 0.87 | 83.9 |
| Soluble proteins (mg . g-mucosa$^{-1}$) | 15 | 6.45 ± 0.52 | 9.5 | 61.38 ± 4.11 | 90.5 |
| CaBP (μg . mg-soluble-protein$^{-1}$) | | 112 | | 66.6 | – |

[a] Agar replica protein content is expressed per g mucosa from which it was removed
[b] Total protein found by adding content of protein in the agar replica to protein which is left in the mucosa

Table 2
Distribution of marker enzyme activities between the mucosa and its agar replica

| Enzyme | Substrate | n | Agar replica | | Mucosa after removing the replica | |
|---|---|---|---|---|---|---|
| | | | Activity | % Total activity in mucosa | Activity | % Total activity in mucosa |
| Lactate dehydrogenase[a] | Lithium lactate | 8 | 0 | 0 | 6.56 ± 0.43 | 100 |
| Invertase[a] | Sucrose | 8 | 0 | 0 | 9.86 ± 0.89 | 100 |
| Alkaline phosphatase[b] | Sodium β-glycerophosphate | 8 | 0.73 ± 0.06 | 15.6 | 3.92 ± 0.23 | 84.4 |

[a] Activity is expressed as μmol . g-mucosa$^{-1}$ . min$^{-1}$
[b] Activity is expressed as μmol $P_i$ . g-mucosa$^{-1}$ . min$^{-1}$

These data indicate that the protein fraction in the agar replica is enriched in CaBP. This is seen more clearly if the case content is expressed per mg of soluble protein (line 3 of table 1).

In table 2 is shown the distribution of activities of marker enzymes between the duodenal mucosa and its agar replica. Lactate dehydrogenase, the cytoplasmic marker enzyme, cannot be detected in the agar replica. The same is true for invertase, which is considered to be a marker of the microvillous membrane. However, alkaline phosphatase is convincingly shown to be present in the replica (15.6% of the total content).

## 4. Discussion

Electron microscopic investigations in [10] showed that forming an agar replica in rat intestinal segments left intact the ultrastructure of the epithelium cells and the brush border. Similar electron micrographs have been obtained for chick intestine in our laboratory [16]. These observations show that agar gel, when removed, takes away in it only the apical part of the glycocalyx situated above the microvilli. At the same time the lateral glycocalyx occupying the intermicrovillous space is not touched by the procedure. Apparently the agar solution is incapable of penetrating the narrow spaces between the microvilli. Therefore agar gel forms a mechanical contact with the apical glycocalyx only which is taken away together with the replica. The fact that activities of the intestinal marker enzymes invertase and lactate dehydrogenase are absent from the replica (table 2) indicates that the integrity of mucosal cells is preserved and no release of cytoplasmic content into the lumen takes place, either during filling of the intestinal segment with agar solution or during the removal of the agar replica.

The presence of CaBP in the replica when enterocytes and their apical membranes remain intact convincingly shows that a certain part of the protein is localized on the surface of the epithelium in the brush border region. No information about the subcellular localization of CaBP not included in the replica is obtained using this method.

Further, the present findings indicate that in the chick intestine 15.6% of the total alkaline phosphatase activity is included in the agar replica (table 2). This fully agrees with results obtained with rats using the same method [10]. Similarly to alkaline phosphatase, 16.1% of the CaBP is removed from the intestinal mucosa by the replica. It should be noted that alkaline phosphatase is not an intracellular enzyme and is localized in the brush border [17]. Apparently this applies also to CaBP.

It is important to note that the portion of CaBP in the agar replica is increased in comparison with the intact duodenal mucosa (112 and 66.6 $\mu$g/mg soluble protein, respectively). This shows that CaBP enters the replica more easily than other intestinal proteins localized in the brush border region. This observation is consistent with the concept that CaBP is not tightly bound to structures in the glycocalyx or to the lipoprotein membrane but rather is dissolved in the water present in the brush border.

Our results obtained using the agar replica method are in good agreement with the data of a series of immunocytochemical investigations. Using immunofluorescence microscopy [7], as well as the electron microscope [8,9] it has been demonstrated that in the intestine of chicks repleted with vitamin $D_3$ CaBP is synthesized by enterocytes and/or goblet cells and is localized in the brush border region of the intestinal epithelium.

Results of our recent investigations [18] show that the low-affinity binding sites of CaBP may have some important role in intestinal calcium absorption. The low-affinity binding sites of CaBP can take an active part in calcium-transport only on the apical surface of the epithelium where $Ca^{2+}$ is $> 10^{-3}$ M. Therefore, presumably, the brush border of the enterocyte is where CaBP should be localized.

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