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Analysis of cytoskeletal proteins and Ca^{2+}-dependent regulation of structure in intestinal brush borders from rachitic chicks

(calcium/cytoskeleton/intestine/microvillus/vitamin D)

CHRISTINE L. HOWE*, THOMAS C. S. KELLER III*, MARK S. MOOSEKER*, AND ROBERT H. WASSERMAN†

*Department of Biology, Yale University, New Haven, Connecticut 06511; and †Department of Physiology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

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ABSTRACT **We have investigated several structural aspects of the intestinal epithelial brush border from rachitic chicks. At both the light and electron microscope levels, rachitic brush borders are indistinguishable from controls. Although several of the prominent periodic acid–Schiff-positive proteins of the brush border membrane have slightly slower mobilities on sodium dodecyl sulfate/polyacrylamide gels than do corresponding proteins from control brush borders, the major components of the microvillus core, including subunits of 105, 95, and 68 kilodaltons, actin, and calmodulin, are not detectably different. As assayed by a ^{125}I-labeled calmodulin gel overlay technique, the same calmodulin-binding proteins are present in rachitic and control brush borders. Two proteins, the 105-kilodalton subunit of the microvillus core and an approximately 30-kilodalton membrane protein, bind calmodulin in a calcium-independent manner. Four cytoskeletal proteins (250, 190, 180, and 150 kilodaltons) and one membrane protein (35 kilodaltons) bind calmodulin only in the presence of calcium. Calcium-dependent solation of microvillus core proteins and calcium-dependent phosphorylation of the 20-kilodalton light chain of brush border myosin both occur as in controls. Our results show that rachitic chicks have brush borders that are quite similar to controls with respect to their ultrastructural organization, constituent contractile proteins, and calcium-dependent regulation of contractility and microvillus core structure. Therefore, the decreased absorption of calcium by intestinal epithelial cells in rachitic chicks is probably not due to gross structural or chemical differences in the brush border cytoskeleton.**

One major function of vitamin D is regulation of the movement of calcium across intestinal epithelium (1–4). In vitamin D deficiency, the absorption of dietary calcium is substantially reduced; administration of vitamin D or its biologically active metabolites stimulates calcium absorption by means that are not yet completely understood. Several observations have been made that should contribute to an understanding of the mechanisms and sites of action of vitamin D on calcium transport at both physiological and molecular levels. These include the induction of the vitamin D-dependent calcium-binding protein (CaBP) (5–8), stimulation of the synthesis of alkaline phosphatase and calcium-stimulated adenosine triphosphatase (9–11), the synthesis of a membrane-bound calcium-binding complex (12), an alteration of lipid components of the brush border membrane (13–16), and an increase in the activity of the acylation–deacylation cycle of brush border phosphatidylcholine (17). Morphological changes in rachitic chick duodenal epithelial cells have also been reported (18). Other vitamin D-dependent changes have been recently summarized (19). The multiple effects of vitamin D in the intestinal cell certainly reflect both direct and indirect (via changes in calcium) actions of this steroid, and these molecular events appear to involve both protein synthetic and non-protein-synthetic events.

Wilson and Lawson (20) reported effects of vitamin D that may specifically involve the cytoskeletal structure of the brush border. They showed that an early response of rachitic chicks to an acute dose of 1,25-dihydroxyvitamin D_3 is the increased rate of synthesis of a 42- to 45-kilodalton (kDal) microvillar protein. This protein was later tentatively identified as β- and γ-actin (21). Whether there is an increased turnover or *de novo* synthesis of the actin-like protein had not been clarified. With the assumption that the 42- to 45-kDal protein is *bona fide* actin, the results of Wilson and Lawson (20, 21) are of considerable interest from the standpoint of both the action of 1,25-dihydroxyvitamin D_3 on calcium transport and the potential effect of the vitamin D hormone on the structure and behavior of the microvillar cytoskeleton. The role of calcium in the regulation of brush border contractility (22), brush border myosin phosphorylation (23), and the length and bundling of microvillar actin filaments (24–29) has been established. Also, the microvillus contains considerable amounts of calmodulin (30), for which only one function, activation of the brush border myosin light chain kinase (23), has as yet been defined. Thus, the significance of calcium in the modulation and regulation of the transmural flow of calcium through the intestinal epithelial cell constituted the basis for the present investigation. The main objective was to determine whether vitamin D alters some of the known molecular components of the microvillus cytoskeleton, and thus to provide the basis for further studies on the relationships among the cytoskeleton of the brush border, vitamin D, and calcium transport. Other observations on the protein components of the brush border are reported herein. A review of brush border structure and function has recently appeared (31).

MATERIALS AND METHODS

Animals. White Leghorn cockerels were maintained on a rachitogenic diet (32) in a room with incandescent lighting for 4–5 weeks. Some chicks were injected with 500 international units of vitamin D_3 at least 72 hr before the experiments were performed and fed a vitamin D-complete diet; these chicks served as vitamin D replete controls. To verify the vitamin D status of animals, radial immunoassays for vitamin D-dependent calcium-binding protein were performed as described (33) on supernates saved from the first cell homogenization step in the preparation of brush borders.

Isolation of Brush Borders and Microvilli. Brush borders were prepared from chick intestinal epithelium by the procedure of Mooseker *et al.* (34). Microvilli were isolated from brush borders by procedures described by Howe *et al.* (30). To control

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Abbreviation: kDal, kilodalton(s).

proteolysis, 0.1 mM α-toluenesulfonyl fluoride (Kodak) and 10–20 trypsin inhibitor units of aprotinin (Sigma) per liter were added to all solutions. For phosphorylation experiments, brush borders were isolated by this method modified to remove contaminating nuclei by floating the brush borders to the 40–50% interface of a sucrose step gradient (wt/vol in solution A: 75 mM KCl/5 mM $MgCl_2$/1 mM EGTA/4 mM NaN_3/0.1 mM dithiothreitol/10 mM imidazole·HCl, pH 7.3). Brush borders and microvilli were demembranated by suspension in 2% Triton X-100 in solution A containing aprotinin at 40–80 trypsin inhibitor units per liter.

Preparation of Partially Purified Calmodulin. Samples of partially purified brush border calmodulin were made from demembranated brush borders as described by Howe *et al.* (30).

Iodination of Calmodulin and ^{125}I-Labeled Calmodulin Overlay Technique. Calmodulin was purified from bovine brain by the procedure of Burgess *et al.* (35). One milligram of purified calmodulin was iodinated by the lactoperoxidase method of Richman and Klee (36), as modified by Carlin *et al.* (37). $Na^{125}I$ was obtained from Amersham. Samples of brush borders and microvilli were electrophoresed on $NaDodSO_4$/4–16% polyacrylamide gradient gels and processed for ^{125}I-labeled calmodulin (^{125}I-calmodulin) overlay by the method of Carlin *et al.* (37).

Solation of Brush Border Core Proteins. Calcium-dependent solation experiments were performed as described by Mooseker *et al.* (28).

Phosphorylation of Proteins in Intact and Demembranated Brush Borders. Pelleted brush borders and Triton-treated brush borders were washed once in 75 mM KCl/1 mM $MgCl_2$/0.1 mM EGTA/10 mM imidazole·HCl (pH 7.0), and aliquots were distributed into small tubes. Phosphorylation was assayed in this same solution with 0.1 mM $CaCl_2$ or 1 mM EGTA added. [γ-^{32}P]ATP stock was made by adding [γ-^{32}P]ATP (2000 Ci/mmol from Amersham; 1 Ci = 3.7×10^{10} becquerels) to a stock of unlabeled ATP (Sigma) to give a final concentration of 0.1 mM ATP (approximately 0.1 Ci/mmol) when diluted 1:10 into the reaction mixture.

Phosphorylation of brush border proteins was determined by suspending individual pellets in the assay solutions without ATP and incubating them for 1 min at room temperature. The reaction was then started by adding [γ-^{32}P]ATP stock. The reaction was stopped at 0.5, 1, 3, and 30 min by removing aliquots, diluting them into gel sample buffer, and immediately boiling them. Samples were electrophoresed on $NaDodSO_4$/4–16% polyacrylamide gradient gels, and the stained gels were dried and autoradiographed, using Kodak XAR-5 x-ray film.

Other Methods. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed by the method of Laemmli (38). Gels were stained by the method of Fairbanks *et al.* (39). Light micrographs of brush borders were taken on Kodak technical pan 2415 film, using Zeiss phase-contrast optics. Fixation of samples for thin-section electron microscopy was performed by the method of Begg *et al.* (40).

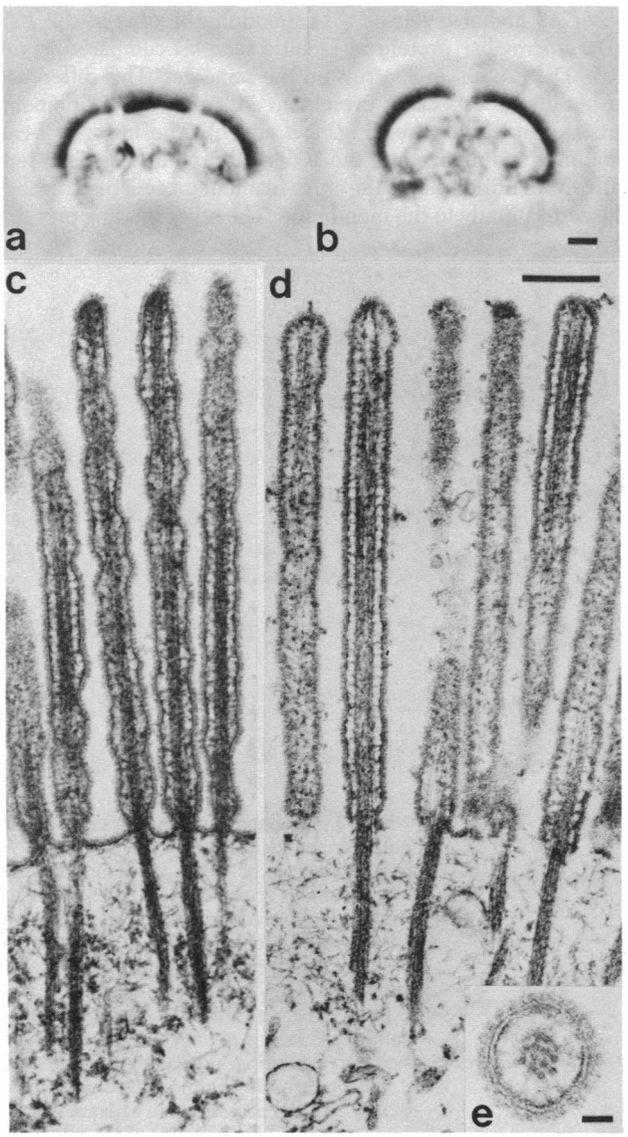

FIG. 1. Morphological comparison of brush borders isolated from rachitic and vitamin D-injected chicks. Light (*a*, *b*) and electron (*c*, *d*, *e*) micrographs of control (*a*, *c*) and rachitic (*b*, *d*, *e*) preparations. (*a* and *b* × 1800, bar represents 2 μm; *c* and *d* × 50,000, bar represents 0.2 μm; *e* × 180,000, bar represents 0.02 μm.)

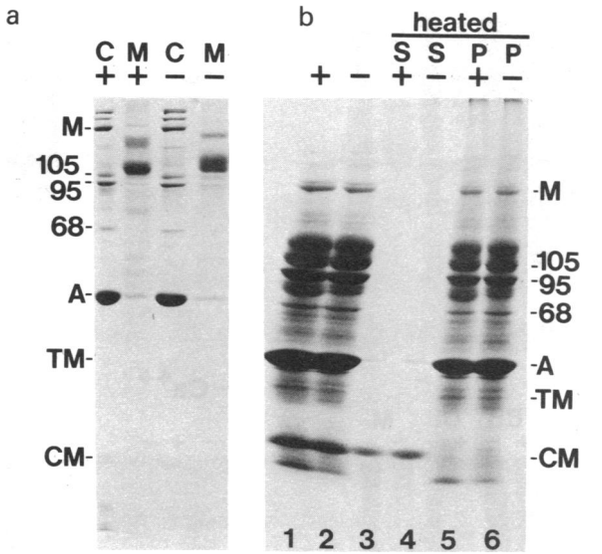

FIG. 2. (*a*) Comparison of brush border proteins: $NaDodSO_4$/10% polyacrylamide gel electrophoresis of Triton-treated brush border cytoskeletons (C) and solubilized membrane fractions (M) from vitamin D-injected (+) and rachitic (−) chicks. Cytoskeletal proteins of both preparations include myosin (M), actin (A), tropomyosin (TM), calmodulin (CM), and subunits of 105, 95, and 68 kDal. (*b*) Purification of brush border calmodulin: $NaDodSO_4$/4–16% polyacrylamide gradient gel electrophoresis of supernate (S, lanes 3 and 4) and pellet (P, lanes 5 and 6) fractions from detergent-treated brush borders after 5-min incubation at 90°C. Lanes 1 and 2 contain the same preparations prior to heat treatment. Calmodulin is released into the supernate fraction in both rachitic and control preparations.

RESULTS

Structure of Brush Borders Isolated from Rachitic Chicks. Structurally, at the light microscope level, brush borders from rachitic chicks are indistinguishable from normal brush borders (Fig. 1). No qualitative differences in either the terminal web region or the microvilli are apparent. However, in several experiments, we noticed the microvilli on brush borders from rachitic chicks were significantly longer than normal microvilli by about 10–25%. The length differences between rachitic and control microvilli were particularly evident when chicks had been fasted overnight.

At the level of the electron microscope, brush borders from rachitic chicks are similar in all respects to the normal structures (Fig. 1). [For review of normal brush border structure, see Mooseker and Howe (31).]

Cytoskeletal Proteins. The protein compositions of brush borders as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis are very similar in rachitic and normal chicks (Fig. 2), although conspicuous differences do exist in the membrane fractions. Several of the prominent, periodic acid–Schiff-positive proteins of the brush border membrane have slightly slower mobility than the corresponding proteins from control brush borders. The demembranated brush borders are also quite similar; some minor bands differ, but the major components of the microvillus core, protein subunits of 105, 95, and 68 kDal, actin, and calmodulin, are apparently identical. As we have shown (30), calmodulin is a major component of the normal brush border microvillus core. The cytoskeletal protein of 17 kDal in rachitic brush borders behaves like the calmodulin in normal brush borders, as indicated by its heat resistance (Fig. 2), comigration on $NaDodSO_4$ gels (Figs. 2 and 4), and susceptibility to solation by Ca^{2+} (Fig. 4).

The interaction of calmodulin with brush border and microvillus proteins is similar in control and rachitic chicks as assayed by a ^{125}I-calmodulin gel overlay technique (Fig. 3). In this procedure, labeled calmodulin binds to proteins first separated on $NaDodSO_4$ gels and then at least partially renatured by dialysis of $NaDodSO_4$ from the gel. Under these conditions, the most prominent binding proteins in terms of exposure density on autoradiograms are the 105-kDal protein of the microvillus and a terminal web protein of 250 kDal, as previously reported (41). In addition to these proteins, we have identified five additional subunits, three associated with the cytoskeletal domain (approximately 190, 180, and 150 kDal), and two associated with the membrane (approximately 35 and 30 kDal). Except for microvillar 105-kDal protein and the smaller of the membrane proteins (approximately 30 kDal), all of these proteins bind ^{125}I-calmodulin only in the presence of Ca^{2+}. Although this technique is not quantitative, we have consistently observed higher levels of ^{125}I-calmodulin binding to the 105-kDal microvillar protein in rachitic samples compared to the 105-kDal protein in controls on the same gel. This difference in binding is especially prominent in samples from demembranated microvilli (see Fig. 3 *d* and *h*). The major ^{125}I-calmodulin binding protein, the 105-kDal protein, has been tentatively identified (42) as the cross-filament protein that attaches the core laterally to the membrane. The other binding proteins

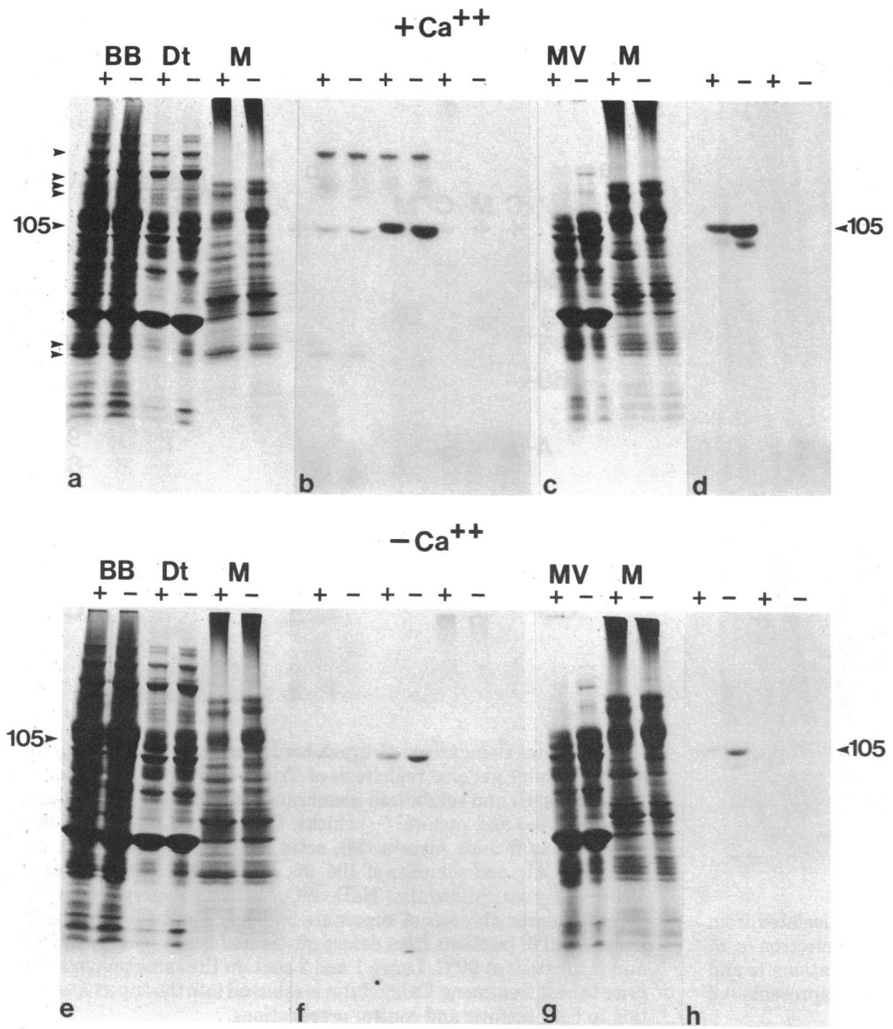

FIG. 3. Calmodulin-binding proteins of isolated brush borders. $NaDodSO_4$/polyacrylamide gels of brush borders (BB), demembranated brush borders (Dt), demembranated microvilli (MV), and respective membrane fractions (M) were incubated with ^{125}I-calmodulin in the presence (*a–d*, $+Ca^{2+}$) or absence (*e–g*, $-Ca^{2+}$) of calcium as described by Carlin *et al.* (37). Coomassie blue-stained gels (*a, c, e, g*) are shown to the left of their corresponding autoradiograms (*b, d, f, h*). The predominant calmodulin-binding protein in both the presence and absence of calcium is the 105-kDal subunit of the microvillus core. Six other binding proteins (arrows) are detected in the brush border or demembranated brush border samples. The most prominent of these is a cytoskeletal protein of 250 kDal that binds calmodulin only in the presence of calcium.

we have observed by using this technique have not been identified, although the band directly under the 105-kDal protein is almost certainly a proteolytic fragment of it (unpublished data).

Calcium-Dependent Regulation of Structure. The reversible, Ca^{2+}-dependent solation of microvillus core proteins, shown by us and others (24–29) to be mediated at least in part by the 95-kDal protein, occurs no differently in microvilli from rachitic chicks (Fig. 4). Another aspect of brush border organization that we have investigated in rachitic chicks is Ca^{2+}-dependent phosphorylation of brush border proteins (Fig. 5). In the absence of Ca^{2+}, a wide range of proteins is rapidly phosphorylated *in vitro* in rachitic brush borders, just as occurs in normal brush borders (23). In the presence of Ca^{2+}, essentially the same proteins are labeled, but there is a marked increase in phosphorylation of the 20-kDal light chain of brush border myosin, again as in controls. One immediately obvious difference between phosphorylation of control and rachitic brush border proteins is the higher relative level of phosphate incorporation that occurs in brush borders (data not shown) and demembranated brush borders (Fig. 5) from rachitic chicks. When autoradiograms are compared in which the relative exposures are matched to compensate for this event, the spectrum of phosphorylated proteins in rachitic brush borders is qualitatively similar to that of the control in both the absence and presence of Ca^{2+}. Except for the 20-kDal light chain of myosin, there is no phosphorylation of other identified cytoskeletal proteins of the brush border, including myosin heavy chain, actin, calmodulin, and the 105-, 95-, and 68-kDal microvillar subunits. The major phosphorylated protein at about 95 kDal is not the 95-kDal subunit of the microvillus, but a protein just below it on the gel.

DISCUSSION

The results of this investigation indicate that no major differences exist in the structure of brush borders from rachitic chicks and from normal animals, at either the light or electron microscope levels. The protein content of the cytoskeletal/contractile apparatus of rachitic brush borders, the reaction of these core proteins to Ca^{2+} in terms of solation and of myosin light chain phosphorylation, and the interaction of calmodulin with brush border proteins are all qualitatively similar to controls. In light of the fact that vitamin D causes a marked increase in the synthesis of brush border actin when administered to rachitic chicks (20), we were in fact surprised at the great similarity between rachitic and control brush borders. These results certainly do not exclude the involvement of the brush border cytoskeletal/contractile apparatus in Ca^{2+} transport. In fact, a recent study demonstrates that cytochalasin B, a drug that inhibits actin assembly (43), decreases active calcium transport by intestinal cells. However, they do demonstrate that the reduced levels of calcium absorption caused by vitamin D deficiency are not due to gross structural or chemical alterations in the cytoskeletal domain of the brush border, although more subtle changes may occur. For example, Wilson and Lawson (20, 21) have shown that vitamin D does stimulate synthesis of brush

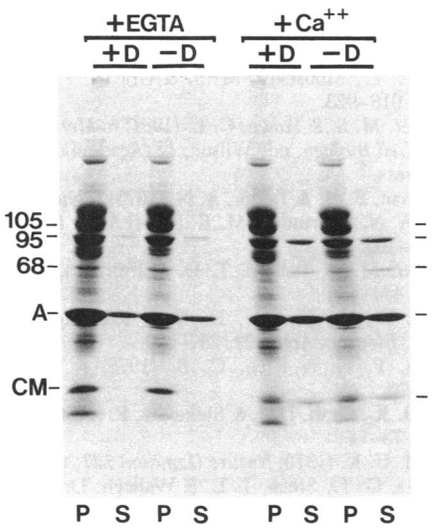

FIG. 4. Calcium-dependent solation of microvillus cores. Microvillar filament bundles from control (+D) and rachitic (−D) brush border preparations were incubated in solutions containing either 1 mM EGTA or 0.1 mM Ca^{2+} for 50 min on ice and 10 min at room temperature. The pellets (P) and supernates (S) after a 100,000 × *g* centrifugation of these suspensions were analyzed by $NaDodSO_4$/4–16% polyacrylamide gradient gel electrophoresis. In the presence of Ca^{2+}, microvillar subunits of 95 and 68 kDal, actin (A), and calmodulin (CM), but not the 105-kDal subunit, are solated and appear in the supernate fraction of both preparations.

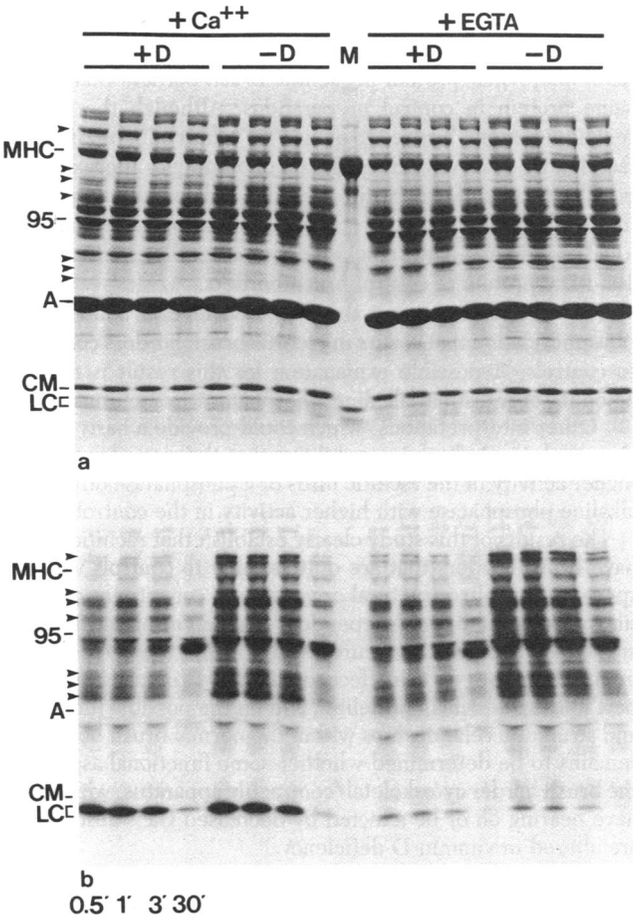

FIG. 5. Calcium-dependent phosphorylation of proteins in demembranated brush borders. Coomassie staining (*a*) and autoradiography (*b*) of a $NaDodSO_4$/4–16% polyacrylamide gradient gel of demembranated brush borders from control (+D) and rachitic (−D) chicks. Samples were incubated for 0.5, 1, 3, and 30 min in [γ-^{32}P]ATP in the presence of 0.1 mM Ca^{2+} (+Ca^{2+}) or 1 mM EGTA (+EGTA). The four lanes under each of the four experimental conditions represent the incubation times. The center lane (M) contains brush border myosin showing the heavy chain (MHC) and two light chains (LC). In the presence of Ca^{2+} there is a rapid and marked increase in the phosphorylation of the 19- to 20-kDal light chain of brush border myosin in both control and rachitic chicks over that observed in the absence of Ca^{2+}. Note that the total level of phosphate incorporation is greater in the preparation from rachitic chicks (see text). Arrows identify corresponding regions on the gels; A, actin; CM, calmodulin; and 95, 95-kDal subunit of the microvillus.

border actin when administered to rachitic chicks. Because we have demonstrated that the relative amount and structural organization of actin in rachitic brush borders is virtually identical to that in control brush borders, a reasonable explanation for their data is a slower rate of actin turnover in rachitic chicks. This notion is supported by our observation that rachitic brush borders have longer microvilli, particularly in fasted animals. In normal animals, fasting induces reversible shortening of microvilli (44–46). Jande and Brewer (18) reported morphological changes in rachitic chick duodenal epithelial cells that include a decrease in length of brush border microvilli. We cannot reconcile our data with theirs and must simply point out differences between our procedures that may help to explain the results. We have used isolated brush borders for our studies, whereas they worked with whole intact epithelial cells. Additionally, we looked at microvillar length at both the light and electron microscope levels, whereas they analyzed length exclusively by electron microscopy, which necessarily limits sample size.

We have observed two obvious differences in our comparison of rachitic and control brush borders. One of these differences is the consistent binding of more ^{125}I-calmodulin to the 105-kDal microvillar protein in rachitic brush borders than to the same protein in control preparations. Although the overlay technique cannot be used to quantitate calmodulin binding, the fact that we observe different levels of binding to approximately equal amounts of protein in the same gel, presumably under identical binding conditions, raises the possibility that the 105-kDal protein in rachitic chicks is not structurally identical to the 105-kDal protein in control brush borders.

The other difference is the increased level of total protein phosphorylation that occurs in rachitic brush borders compared to controls. A possible explanation for this result is reduced amounts of intestinal alkaline phosphatase in rachitic animals (9). Other interpretations, which could provide a basis for further study, include the possibilities that there is a kinase with higher activity in the rachitic birds or a phosphatase other than alkaline phosphatase with higher activity in the control birds.

The results of this study clearly establish that rachitic chicks have brush borders that are quite similar to controls with respect to their ultrastructural organization, constituent contractile proteins, and Ca^{2+}-dependent regulation of contractility and microvillus core structure. Additionally, they give further information both on the effects of vitamin D on calcium transport in the intestinal epithelium and on the possible functional and structural relationships within the normal brush border. It remains to be determined whether some functional aspects of the brush border cytoskeletal/contractile apparatus, which may have bearing on or be affected by decreased Ca^{2+} absorption, are altered in vitamin D deficiency.

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