Cysteine-rich intestinal protein binds zinc during transmucosal zinc transport

(trace element/intestinal absorption/metal binding/LIM motif/developmental regulation)

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ABSTRACT   The mechanism of zinc absorption has not been delineated, but kinetic studies show that both passive and carrier-mediated processes are involved. We have identified a low molecular mass zinc-binding protein in the soluble fraction of rat intestinal mucosa that could function as an intracellular zinc carrier. The protein was not detected in liver or pancreas, suggesting a role specific to the intestine. The protein binds zinc during transmucosal zinc transport and shows signs of saturation at higher luminal zinc concentrations, characteristics consistent with a role in carrier-mediated zinc absorption. Microsequence analysis of the protein purified by gel-filtration HPLC and SDS/PAGE showed complete identity within the first 41 N-terminal amino acids with the deduced protein sequence of cysteine-rich intestinal protein [Birkenmeier, E. H. & Gordon, J. I. (1986) Proc. Natl. Acad. Sci. USA 83, 2516–2520]. These investigators showed that the gene for this protein is developmentally regulated in neonates during the suckling period, conserved in many vertebrate species, and predominantly expressed in the small intestine. Cysteine-rich intestinal protein contains a recently identified conserved sequence of histidine and cysteine residues, the LIM motif, which our results suggest confers metal-binding properties that are important for zinc transport and/or functions of this micronutrient.

Dietary Zn is absorbed primarily by the small intestine, but the mechanism(s) has not been identified (1–3). The process may be energy dependent (4, 5) and is inversely related to the intracellular concentration of intestinal metallothionein (2, 6), a low molecular mass protein that binds Zn, Cu, Cd, and other trace elements. Kinetic analyses have shown that Zn absorption includes both passive diffusion and carrier-mediated components (7–9) that may represent paracellular and transcellular absorption pathways. Carrier-mediated Zn absorption is increased in Zn-deficient animals, suggesting up-regulation of the carrier system (7, 9).

Using gel-filtration HPLC, we identified a low molecular mass, intracellular constituent from rat intestinal mucosa that binds Zn during transmucosal Zn transport (10). It was not metallothionein, based on Cd–hemoglobin affinity assay. When ^{65}Zn was introduced into ligated duodenal loops, the proportion of intracellular ^{65}Zn associated with this constituent was reduced when chelating agents that reduced Zn absorption were also present. This suggested a functional role in Zn absorption.

In the present study, we used a combination of gel-filtration HPLC, SDS/PAGE, and Western blotting to show that the ^{65}Zn-binding constituent is a protein that is present in the intestine but not in liver or pancreas. The amino acid sequence of the purified protein is identical to the deduced sequence of cysteine-rich intestinal protein (CRIP), encoded

by a developmentally regulated mRNA identified by Birkenmeier and Gordon (11). Our data suggest that CRIP has a role in Zn absorption and may function as an intracellular Zn transport protein.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley strain rats (Harlan Sprague–Dawley) weighing 150–200 g were maintained as described (10). Tap water and a commercial, nonpurified diet (Rodent Laboratory Chow 5001, Ralston Purina) containing ≈70 mg of Zn per kg were supplied ad libitum.

Sample Preparation. Intestinal proteins were labeled with ^{65}Zn by introducing 0.5 ml of saline containing 185 kBq of ^{65}Zn (DuPont/NEN, 68 kBq of ^{65}Zn per nmol of Zn) into 10-cm ligated loops of the proximal small intestine of anesthetized rats (10). The Zn concentration of the injected solution was adjusted when necessary by adding $ZnSO_4$. After 15 min, the segment was drained and rinsed with ice-cold homogenization buffer [10 mM Tris·HCl (pH 8.6) containing 154 mM NaCl, 0.02% NaN_3, 0.2 mM phenylmethylsulfonyl fluoride, and 0.6 μg of leupeptin and 0.9 μg of pepstatin A per ml], and the mucosal layer was collected. Samples of liver and pancreas were labeled for 15 min with ^{65}Zn by injecting 1100 kBq of ^{65}Zn into the hepatic portal vein. Homogenates of liver, pancreas, and intestinal mucosa were prepared 1:3 (wt/vol) in homogenization buffer by using a Potter–Elvehjem, glass–Teflon tissue grinder. After centrifugation at $40,000 \times g$ for 30 min at 4°C, the supernatant fraction (hereafter referred to as cytosol) was filtered (0.2 μm), and the soluble proteins were separated by gel-filtration HPLC.

Gel-Filtration HPLC. Samples were eluted from two 1 × 30 cm Superose 12 columns (Pharmacia) connected in series at 0.5 ml/min [≈400 psi (1 psi = 6.89 kPa) with 10 mM Tris·HCl (pH 8.6) containing 154 mM NaCl, 10 mM $MgSO_4$, and 0.02% NaN_3]. HPLC fractions (0.5 ml) were measured for ^{65}Zn by γ spectrometry, total Zn was assayed by air–acetylene flame atomic absorption spectrophotometry (10), and metallothionein concentration was determined by Cd–hemoglobin affinity assay (12). All chromatographic profiles shown are representative of three or more runs of samples from different animals.

SDS/PAGE. Selected HPLC fractions were concentrated by ultrafiltration ($M_r > 3000$; Centricon-3, Amicon), and the proteins were resolved further by discontinuous SDS/PAGE on 15% Tris–tricine/polyacrylamide gels (13). The concentrated fractions were mixed 1:1 (vol/vol) with 125 mM Tris·HCl (pH 6.8) containing 4% (wt/vol) SDS, 20% (vol/vol) glycerol, and 10% (vol/vol) 2-mercaptoethanol and were

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Abbreviations: CRIP, cysteine-rich intestinal protein; V_e, elution volume.
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heated 5 min at 100°C. Electrophoresis was conducted at a constant current (30 mA per gel) with cooling by tap water. Molecular mass (kDa) standards (Life Technology, Grand Island, NY) were ovalbumin (45.4), carbonic anhydrase (29.1), β-lactoglobulin (18.1), lysozyme (14.4), bovine trypsin inhibitor (5.8), and insulin (3.0).

Western Blotting–^{65}Zn Binding Assay. Proteins resolved by SDS/PAGE were transferred at 0°C to poly(vinyl difluoride) or 0.2-μm nitrocellulose membranes (Schleicher & Schuell) at 12 V for 3 hr in 10 mM 2-(4-morpholino)ethanesulfonic acid (pH 6.0) containing 20% (vol/vol) methanol. Prior to labeling the immobilized proteins with ^{65}Zn, the nitrocellulose was equilibrated 2 hr in 10 mM Tris·HCl (pH 7.5) containing 1 mM MnCl$_2$ and 10 mM 2-mercaptoethanol. The membrane was then equilibrated for 15 min in 50 ml of buffer containing 37 kBq of ^{65}Zn per ml. Residual radioisotope was removed by rinsing the membrane at least three times for 10 min in 200 ml of buffer without the MnCl$_2$. ^{65}Zn-binding proteins on the dried nitrocellulose were visualized by autoradiography and then stained for total protein with amido black.

Protein Sequence Analysis. Proteins transferred to poly(vinyl difluoride) membranes were visualized by staining with 0.01% Coomassie brilliant blue R in 50% methanol containing 10% acetic acid. Selected protein bands were excised, and their amino acid sequence was determined by Edman degradation on a model 473A gas-phase protein sequencer with model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems) at the University of Florida Protein Chemistry Core Facility. The resulting sequence was compared to those listed in a computer data base by using the MICROGENIE protein and gene sequence analysis program (Beckman).

RESULTS

Identification of a Zn-Binding Protein Specific to the Intestine. A 6- to 9-kDa ^{65}Zn peak was identified in the intestinal mucosal cytosol by gel-filtration HPLC (Fig. 1). A comparable peak was not detected in cytosol from either liver or pancreas. The peak did not comprise metallothionein, which was eluted earlier and was observed in all three tissues. A large proportion of the intracellular ^{65}Zn from the intestinal mucosa was associated with this peak 15 min after ^{65}Zn was introduced into the lumen. This suggests that a constituent eluting in this peak avidly binds Zn during transmucosal Zn transport.

Fractions eluting at the same volume ($V_e = 30.5$–32.5 ml) as the intestinal ^{65}Zn peak were collected from each tissue, further resolved by SDS/PAGE, and transferred onto nitrocellulose. Staining with amido black and labeling with ^{65}Zn (Fig. 2) confirmed that the intestine contained a Zn-binding protein with an apparent molecular mass similar to that determined by gel-filtration HPLC and that the protein was not present in liver or pancreas.

Protein Sequence Analysis and Identification as CRIP. Intestinal proteins resolved by SDS/PAGE as in Fig. 2 were transferred to poly(vinyl difluoride) membrane for microsequence analysis. We were able to identify 37 of the first 41 N-terminal amino acids from the excised protein band corresponding to the intestinal Zn-binding protein. Computer search of a protein and gene sequence data base showed that, except for the lack of a terminal methionine, the sequence of our intestinal Zn-binding protein (Fig. 3) was identical to that of the deduced sequence of a protein encoded by a developmentally regulated gene identified by Birkenmeier and Gordon (11). They named the protein CRIP because the mRNA coding for this protein was most abundant in the small intestine, and because the 77 amino acid protein contained 7 (9%) cysteine residues.

Role of CRIP in Zn Absorption. The specific presence of CRIP in the intestine suggests a role in nutrient absorption.
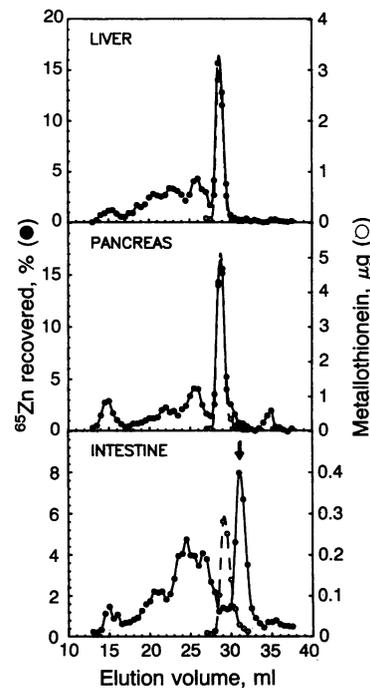

FIG. 1. Distribution of ^{65}Zn among intracellular constituents of cytosol separated by gel-filtration HPLC showing the presence of a low molecular mass ^{65}Zn peak specific to the intestine (designated by the arrow in *Bottom*) ($V_e = 31.5$ ml). Liver (*Top*) and pancreas (*Middle*) cytosol were labeled with ^{65}Zn injected into the hepatic portal vein. Intestinal mucosa was labeled with ^{65}Zn introduced into the lumen of a ligated segment of the proximal small intestine. The elution volume of metallothionein ($V_e = 29.5$ ml) was determined by Cd–hemoglobin affinity assay.

Since CRIP binds Zn during transmucosal Zn transport, CRIP could function as an intracellular Zn carrier. Kinetic studies have shown that Zn absorption involves a carrier-mediated mechanism that is saturable at luminal Zn concentrations between 60 and 100 μM (7, 9). Insight into the

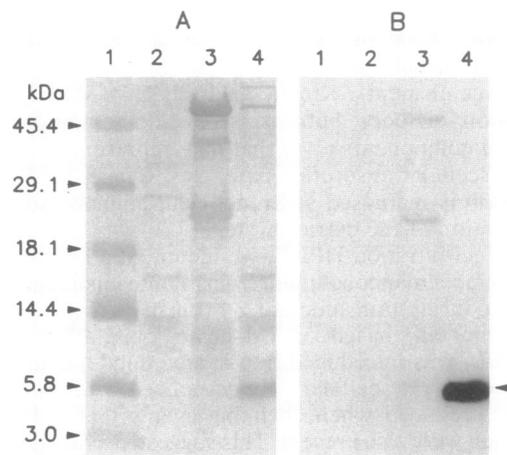FIG. 2. Western blotting and ^{65}Zn labeling of low molecular mass proteins showing the presence of an intestinal ^{65}Zn-binding protein (designated by the arrowhead on the right) that is absent in liver and pancreas. The constituents in cytosol from each tissue were first separated by gel-filtration HPLC as in Fig. 1. Fractions at the elution volume of the major ^{65}Zn peak in intestinal cytosol ($V_e = 30.5$–32.5 ml) were collected from all three tissues, further resolved by SDS/PAGE, and then transferred to nitrocellulose. The membrane was labeled with ^{65}Zn to produce an autoradiograph (*B*) and then stained with amido black (*A*). Lanes: 1, molecular mass standards; 2, liver cytosol; 3, pancreas cytosol; 4, intestinal mucosal cytosol.

Biochemistry: Hempe and Cousins

Proc. Natl. Acad. Sci. USA 88 (1991) 9673

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M P K C P K C D K E V Y F A E R V T S L G K D W H R P C L K C E K C G K T L T S

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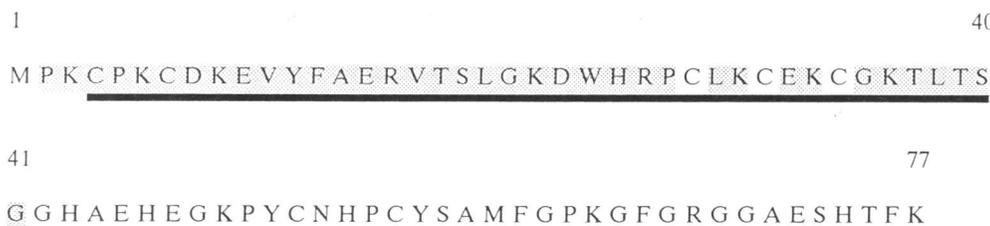G G H A E H E G K P Y C N H P C Y S A M F G P K G F G R G G A E S H T F K

FIG. 3. Sequence homology of the intestinal Zn-binding protein and the deduced sequence of CRIP. The complete deduced sequence of CRIP (11) is shown. Residues of the intestinal Zn-binding protein identified by microsequencing are denoted by shading. Residues comprising the LIM motif are underlined with a bar.

possible role of CRIP as a Zn transport protein was obtained by evaluating the intracellular distribution of total Zn and of ^{65}Zn when the luminal Zn level was above or below this saturation concentration (Fig. 4). Since the ^{65}Zn was introduced directly into the lumen of the intestine, it served as a tracer for the movement of luminal Zn during transmucosal transport.

CRIP bound nearly half of the Zn entering the cells from the lumen [42 ± 4% (SD) of the ^{65}Zn] but only 11 ± 2% (SD) of the total Zn in the cytosol (measured by atomic absorption spectrophotometry) when the luminal Zn concentration was 5 μM—i.e., below the saturation concentration for carrier-mediated Zn absorption (Fig. 4). The higher specific activity of CRIP compared with the average of all other Zn-binding constituents in the cytosol (1.15 vs. 0.18 Bq of ^{65}Zn per pmol of Zn) suggests that the greater accumulation of ^{65}Zn by CRIP represents a much greater interaction with ^{65}Zn entering the cell from the lumen, as might be expected of a carrier protein. In contrast, when the luminal Zn concentration was 300 μM, or well above the saturation concentration for carrier-mediated Zn absorption (Fig. 4), CRIP bound only 25 ± 2% (SD) of the ^{65}Zn in the cytosol. The lower proportion of ^{65}Zn associated with CRIP at the higher luminal Zn concentration could indicate that carrier-mediated Zn transport was saturated and that excess Zn entering the cell from the lumen was associated with intracellular constituents other than the carrier protein. This is supported by the observation that neither

total Zn (Fig. 4) nor the proportion of total intracellular Zn (9 ± 2% SD) bound to CRIP was different at the higher luminal Zn concentration, suggesting that CRIP has a limited Zn-binding capacity. The specific activity of CRIP was still greater than the average of all other intracellular Zn-binding constituents (0.28 vs. 0.08 Bq of ^{65}Zn per pmol of Zn), indicating greater interaction with luminal Zn even at the higher luminal Zn concentration.

DISCUSSION

Birkenmeier and Gordon (11) showed that CRIP mRNA is most abundant in adult rodent small intestine and more abundant in villus than crypt cells. Similar levels of CRIP mRNA were observed in duodenum, jejunum, and ileum, while the colon contained 46% of that observed in the small intestine. Much less was reported in other tissues compared with that in the small intestine, including lung (15%), spleen (12%), adrenal (9%), and testis (8%). CRIP mRNA was not detected in brain, kidney, or liver. The CRIP gene sequence is conserved in many vertebrate species and expressed specifically in the intestine, indicating a fundamental role in nutrient absorption or intestinal metabolism. Our data show that CRIP binds Zn during transmucosal Zn transport and could be involved in carrier-mediated Zn absorption.

CRIP may function as an intracellular Zn carrier with a role analogous to that proposed for calbindin in calcium absorption. Calbindin purportedly facilitates calcium absorption (14, 15) by competitively binding calcium at the brush border, increasing the intracellular concentration of diffusible calcium (i.e., calcium that moves through the cell to the basolateral membrane). Parallels between Zn and calcium absorption kinetics and between CRIP and calbindin, respectively, suggest that calcium and Zn absorption mechanisms may have some similar characteristics. Both nutrients are absorbed by passive and carrier-mediated transport mechanisms (7, 9, 14), and both carrier-mediated mechanisms are developmentally regulated (14, 16). Intestinal levels of CRIP mRNA (11) and calbindin (14) reflect these developmental changes in carrier-mediated transport, suggesting that CRIP, like calbindin, could have a functional role in mineral absorption.

The developmental regulation of CRIP and its possible role in Zn absorption suggest an involvement in acrodermatitis enteropathica, a human autosomal recessive Zn malabsorption disease (17). Symptoms of severe Zn deficiency are usually observed in afflicted infants shortly after weaning from breast milk. Although the etiology of the disease is unknown, the deficiency can be alleviated by oral Zn supplementation, indicating a fundamental defect in Zn transport. Evidence of dysfunctional CRIP synthesis or metabolism in persons or animals with acrodermatitis enteropathica or other diseases expressing Zn malabsorption would be positive indication of a role for CRIP in Zn absorption.

The deduced protein sequence of CRIP contains a histidine residue and repeated cysteine residues characteristic of the
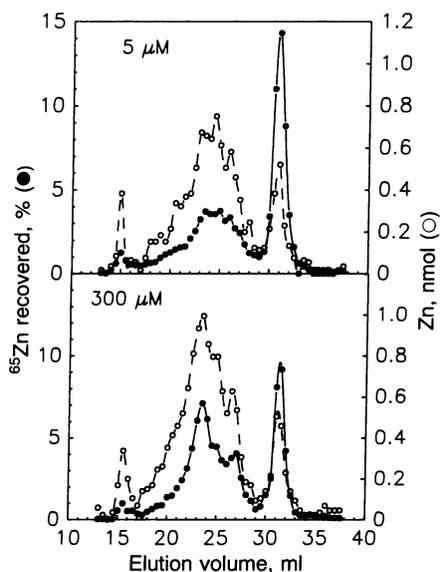

FIG. 4. Distribution of ^{65}Zn and total Zn among intracellular constituents from intestinal mucosa determined by gel-filtration HPLC. Tissues were collected 15 min after ^{65}Zn (tracer for the movement of luminal Zn) was introduced into ligated loops of proximal small intestine at luminal Zn concentrations of 5 and 300 μM. CRIP is the Zn-binding constituent at V_e = 31.5 ml.

novel, cysteine-rich LIM motif [Cys-Xaa$_2$-Cys-Xaa$_{17-19}$-His-Xaa$_2$-Cys-Xaa$_2$-Cys-Xaa$_2$-Cys-Xaa$_{7-11}$-(Cys)-Xaa$_8$-Cys] recently described by Freyd et al. (ref. 18; named LIM for the same cysteine-rich motif of lin-11, isl-1, and mec-3 genes). Because of the metal-binding attributes likely afforded by the cysteine and histidine residues, they suggested that the LIM motif has a metal-binding function similar to that of the consensus motifs of Zn fingers in transcription factors. Our data demonstrate that CRIP binds Zn and strongly supports a metal-binding function for the LIM motif. CRIP differs from other LIM proteins (18–20) because it contains one copy of the motif rather than two and also lacks a homeodomain that would promote binding to DNA. The function of the LIM motif is unknown, but presumably it stabilizes protein-protein and/or protein–nucleic acid interactions (18). Our results suggest that the LIM motif in CRIP could facilitate the protein's interaction with Zn atoms during transport. The resulting conformational change could facilitate transcellular Zn movement or promote site recognition for interaction with other specific components of the Zn transport mechanism. Stoichiometry of Zn binding remains to be determined when more of the protein is available. The LIM motif provides seven cysteine residues and one histidine residue. These ligands could produce Zn "finger," "cluster," and/or "twist" structures (21). A conservative estimate would be two or three Zn atoms per molecule of CRIP. Alternatively, the similarity of the CRIP sequence to bacterial ferredoxins was noted (11). This could suggest a role in binding of other metals through sulfur cluster formation.

Zeng et al. (22) have shown that thionein (apometallothionein) can inactivate binding of the transcription factor Sp1 to DNA. They propose that, because thionein has a higher association constant for Zn than does the Zn finger region of Sp1, inactivation occurs through competition for Zn between Sp1 and thionein. A similar relationship between CRIP and thionein, wherein thionein would remove Zn from CRIP, could explain the proposed regulatory effect of metallothionein on Zn absorption (1–3). We observed that CRIP bound Cd when ^{109}Cd was used instead of ^{65}Zn in ligated duodenal loops or for labeling proteins on nitrocellulose (data not shown). Unlike metallothionein, however, CRIP did not bind ^{109}Cd in competition with hemoglobin when gel filtration HPLC fractions were evaluated by Cd–hemoglobin affinity assay for metallothionein (Fig. 1). This indicates that the association constants for Cd are of the order metallothionein > hemoglobin > CRIP and suggests that thionein probably has a higher association constant for Zn as well. If so, metallothionein may limit Zn absorption by competitively binding Zn in the intestine to inhibit transcellular Zn transport by CRIP.

The identification of CRIP as a major low molecular mass Zn-binding constituent from rat intestinal mucosa has important implications for both past and future research of intestinal Zn metabolism and function. Prior studies (23–26) have identified low molecular mass intestinal Zn-binding constituents that our data now suggest could have included CRIP. Reevaluation of the conclusions of these and similar studies might provide new insight into the biological function or regulation of CRIP. Furthermore, we have observed that metallothionein and CRIP comigrate when analyzed by low-resolution gel-filtration chromatography. This demonstrates that measurement of changes in the elution of Zn or Cd in low molecular mass chromatography fractions is an inappropriate assay for either CRIP or metallothionein. Regulation of intestinal metallothionein synthesis by Zn has been confirmed by amino acid analysis (27) and by changes in metallothionein mRNA (28). However, other studies that have used only gel-filtration chromatography to measure changes

in intestinal metallothionein must be reevaluated, since the reported results could reflect changes in metallothionein, CRIP, or both proteins.

The role of CRIP in Zn metabolism or a role of Zn in CRIP function must be confirmed. Our data show that CRIP interacts avidly with Zn during transmucosal Zn transport and suggest a role in carrier-mediated Zn absorption. A role in Zn absorption, homeostasis, or function will be strengthened by evidence that expression of the CRIP gene is regulated by Zn status, other nutrients, or hormonal factors.

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