Purification and properties of rat cysteine-rich intestinal protein

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Cysteine-rich intestinal protein (CRIP) is a zinc-binding protein where the binding domain is in the so-called LIM double zinc finger motif. Methods are described for the preparation of CRIP from rat small intestine. Gel-filtration and ion-exchange chromatography and preparative PAGE gave homogeneous CRIP, based upon analytical PAGE, mass spectrometry and microsequencing. Initial localization of CRIP during chromatography was based on binding of ^{65}Zn radioisotope introduced into the intestine. The stoichiometry of binding by CRIP is less than 2 atoms of zinc per molecule. The metal-binding affinity *in vitro* is zinc > cadmium > copper > iron, at low metal

concentrations. Zinc is the predominant metal bound when these metals are taken up from the intestinal lumen. Zinc binding was not influenced by pH between values of 4.5 to 7.5. Metallothionein has a much greater zinc-binding affinity than CRIP. The tissue concentration of CRIP is of the order of 15–20 $\mu\text{g/g}$ of mucosal tissue, suggesting that the protein is more abundant than zinc-finger-containing transcription factors. The metal-binding properties of CRIP are consistent with proposed zinc-related functions for this cytoplasmic protein, which is expressed in the small intestine during the postnatal period.

INTRODUCTION

High-resolution gel-filtration chromatography and microsequencing has established that, in the rat small intestine, cysteine-rich intestinal protein (CRIP) binds zinc in a manner that would be expected of a trafficking protein involved in transcellular ion movement (Hempe and Cousins, 1991, 1992). Furthermore, CRIP contains a single copy of the cysteine-rich domain known as the LIM motif. The LIM motif probably accounts for the metal-binding properties of CRIP, but may also provide functional properties that could include protein–protein interactions as well as DNA or RNA binding (Freyd et al., 1990; Sadler et al., 1992).

CRIP expression is developmentally regulated (Birkenmeier and Gordon, 1986; Levenson et al., 1993), increasing between birth and weaning to full adult levels. This developmental expression coincides with the transition from milk to solid food, and could relate to the plethora of changes the intestine undergoes during this period.

To study the biological aspects of CRIP, it is essential to establish a purification method for native CRIP which provides high efficiency and purity. The experiments in this paper describe purification schemes for CRIP from rat small intestine, and define some of the metal-binding properties of this zinc-binding intestinal protein.

MATERIALS AND METHODS

Materials and radioisotopes

Unless noted otherwise, chemicals and materials were purchased from Sigma Chemical (St. Louis, MO, U.S.A.) or Fisher Scientific (Pittsburgh, PA, U.S.A.). ^{65}Zn (3.2 mCi/mg) and ^{64}Cu (2.0 mCi/mg) were from Du Pont NEN (Boston, MA, U.S.A.). ^{65}Zn (carrier-free) was from Amersham (Arlington Heights, IL, U.S.A.), and ^{109}Cd (carrier-free) was from Isotope Products (Burbank, CA, U.S.A.). Chromatography materials were from Pharmacia LKB (Piscataway, NJ, U.S.A.).

Animals

Male Sprague–Dawley strain rats (100–150 g) were given free access to a normal commercial diet (Teklad; 22/5 Rodent 8640 Diet; 70 mg of Zn/kg) and tap water. Housing was in suspended stainless steel cages with a 12 h light/dark cycle, maintained at 22–25 °C. Care and treatment of the rats received prior institutional approval and followed National Institutes of Health guidelines.

Analytical methods

Air/acetylene atomic absorption spectrophotometry (AAS) was used to measure zinc concentrations in chromatographic fractions and solutions. Autoradiography, γ -radiation scintillation counting and liquid scintillation counting were used to detect radioactivity. Protein was measured by various methods: concentrated samples by the method of Lowry et al. (1951), chromatographic fractions by absorbance at 280 nm, and fractions from the preparative PAGE by the method of Bradford (1976). BSA was used as the standard for protein assays. Amino acid sequencing was performed with a gas-phase protein sequencer as described earlier (Hempe et al., 1991). For sequencing after electrophoresis, CRIP was transferred to poly(vinylidene difluoride) (PVDF) membranes (Immobilon-P; 0.45 μm ; Millipore, Bedford, MA, U.S.A.). The mass spectrum was recorded using time-of-flight matrix assisted laser desorption ionization.

In vitro zinc blot assay of ^{65}Zn binding

Aliquots of each fraction collected from chromatography steps or of purified protein were dot-blotted on to a 0.2 μm nitrocellulose membrane using a vacuum. The membrane was incubated in 10 mM Tris/HCl (pH 7.5) containing 1 μCi of $^{65}\text{Zn}/\text{ml}$ as described previously (Hempe and Cousins, 1991). Autoradiography (Kodak X-Omat RP film) used an overnight exposure to determine the zinc-binding fractions. Separate blots

Abbreviations used: AAS, atomic absorption spectrophotometry; CRIP, cysteine-rich intestinal protein; DTT, dithiothreitol; MT, metallothionein; PVDF, poly(vinylidene difluoride).

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were also probed with 1 μ Ci of ^{64}Cu , ^{109}Cd or ^{59}Fe instead of ^{65}Zn .

Gel electrophoresis

Samples were concentrated by ultrafiltration (3000 M_r cut-off; Centricon-3; Amicon, Beverly, MA; or 1000- M_r cut-off; Microsep; Filtron, Northborough, MA, U.S.A.) and the proteins were resolved by discontinuous SDS/PAGE on a 15% Tris/tricine polyacrylamide gel (4% stacking gel) as described by Hempe and Cousins (1991). The proteins were electrophoretically transferred at 12 V for 3 h on to a 0.4 μm PVDF membrane in Mes buffer (10 mM of Mes, pH 6.0, containing 20% methanol) for microsequencing, or a 0.2 μm nitrocellulose membrane in Tris/glycine [25 mM Tris/HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3] transfer buffer for metal-binding studies. The membrane was then subjected to the ^{65}Zn binding assay as described above.

Purification of CRIP

The rats were anaesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL, U.S.A.) and killed by exsanguination. The entire length of intestine was excised and the mucosal layer was removed and homogenized in 10 mM Tris/HCl, 10 μM ZnSO_4 , 154 mM NaCl, 0.02% NaN_3 , 2 mM phenylmethanesulphonyl fluoride, 0.9 $\mu\text{g}/\text{ml}$ pepstatin A, 0.6 $\mu\text{g}/\text{ml}$ leupeptin and 10 mM 2-mercaptoethanol (Hempe and Cousins, 1991). The homogenate was centrifuged at 40000 g for 30 min at 4 $^\circ\text{C}$. The cytosol fraction (supernatant) (five rat intestines per batch) was concentrated by ultrafiltration (YM-2 membrane; Amicon) and applied to a Sephadex G-75 gel-filtration column (2.5 cm $\times$ 90 cm) for the initial fractionation. Elution was with 10 mM Tris/HCl buffer, 10 μM ZnSO_4 and 0.02% NaN_3 , pH 8.0. The zinc concentration of the fractions was measured.

CRIP was detected initially by radiolabelling with ^{65}Zn (1 μCi) via injection of the radioisotope into a ligated loop of the duodenum (Hempe and Cousins, 1991). For these experiments, rats were anaesthetized with pentobarbital sodium (60 mg/kg body wt., intraperitoneal). CRIP was differentiated from metallothionein (MT) by performing the same procedure with intestines from rats where MT was induced by zinc administration [two intraperitoneal injections of 1 mg of Zn(II) at 24 h intervals; rats killed 24 h later]. The radioactive peak in the lower- M_r region contains primarily CRIP in the untreated rats and primarily MT in the zinc-treated rats (Hempe and Cousins, 1992). Routinely, purification of CRIP was carried out using rats that were not administered zinc. Fractions were blotted on to nitrocellulose and assayed by an *in vitro* ^{65}Zn -blot assay as described earlier. The fractions containing CRIP were concentrated by ultrafiltration (YM-2 membranes), and used for gel-filtration f.p.l.c. (Superdex 75 Hiload (16/60) column; Pharmacia LKB). The isocratic elution was carried out with 10 mM Tris/HCl, 154 mM NaCl, 0.02% NaN_3 , 10 mM MgSO_4 , 10 μM ZnSO_4 , pH 8.0, at a flow rate of 0.5 ml/min. CRIP-containing fractions were detected by the zinc blot assay.

SDS/PAGE was sufficiently effective in separating CRIP from other intestinal proteins after a gel-filtration step with Superose 12 f.p.l.c. columns to allow sequencing and identification (Hempe and Cousins, 1991). For that reason, preparative SDS continuous elution PAGE (Model 491 Prep Cell; Bio-Rad, Richmond, CA, U.S.A.) could be used as a final purification step for CRIP. Approx. 2–3 mg of protein was loaded on the discontinuous cylindrical gel (4% stacker, 0.125 M Tris, pH 6.8/15% sep-

arator, 0.375 M Tris, pH 8.8). Electrophoresis, using a Tris/glycine/SDS buffer system (pH 8.3), was carried out at a constant current of 40 mA through the stacker and 55 mA for the separation. Fractions were collected after 4 h of operation. CRIP-containing fractions were concentrated using the Centricon-3 ultrafiltration apparatus and resolved by analytical slab SDS/PAGE (as above) and silver staining (Heukeshoven and Dernick, 1985; Merrill, 1986). The protein was also electrophoretically transferred to a PVDF membrane for microsequencing to confirm its identity as CRIP. The fractions containing CRIP were concentrated as above and dialysed against 10 mM Tris/HCl, 2 mM dithiothreitol (DTT), pH 7.5, using a dialysis membrane (3000- M_r cut-off) and stored at -20°C . All *in vitro* characterization studies were carried out with CRIP prepared by this method.

An alternative method for the preparation of CRIP omitted the use of Superdex 75 Hiload chromatography and replaced continuous PAGE with CM-Sephadex C-25 ion-exchange chromatography (Pharmacia) as the final step. CRIP-containing samples from the low-resolution gel-filtration (G-75 column) step, using 10 mM potassium phosphate buffer at pH 8.0 and 10 μM ZnSO_4 , were eluted from the CM-Sephadex column (2.6 cm $\times$ 15.0 cm) with a 0–0.35 M KCl gradient. CRIP fractions were detected by AAS. Fractions from several CM-Sephadex runs were pooled, concentrated and resolved using Superdex 75 Hiload chromatography to give the final product. Verification of purity was again by SDS/PAGE and microsequencing.

Effect of pH on ^{65}Zn binding by CRIP

Zinc binding was estimated by a method modified from that of Conrad et al. (1990). Aliquots of CRIP (0.4 mg/ml) were incubated with 0.1 μCi of ^{65}Zn in 50 mM Tris/acetate at pH 3.5, 4.5 and 5.5, or in 50 mM Hepes at pH 7.5, 8.5 and 9.5, for 30 min on ice. Following incubation, the solution was diluted with 4 vol. of 50 mM Hepes (pH 7.5) and subjected to ultrafiltration using Centricon-3 microconcentrator tubes (3600 rev./min, 20 min, r_{av} . 6.5 cm, 4 $^\circ\text{C}$). The filtrate contained unbound ^{65}Zn . This was used to obtain the amount of radioactive zinc bound by CRIP. Buffer without the protein was used to correct for ^{65}Zn retained by the microconcentrator tubes. The data were expressed as percentages of the total radioactivity bound.

Competitive binding *in vitro*

A modification of the method of Richardt et al. (1986) was used to study the ability of metal ions to compete with zinc for binding to CRIP. Initially, CRIP (20 μg) was vacuum dot-blotted on to nitrocellulose. The dots were separated and incubated individually at room temperature following a wash with buffer (10 mM Tris/HCl, 1 mM MnCl_2 , 10 mM 2-mercaptoethanol, pH 7.5) for 30 min. For each assay, one nitrocellulose dot was added to a solution containing 10^{-3} , 10^{-4} , 10^{-5} or 10^{-6} M Cu(II) or Cd(II) in 10 mM Tris/HCl with 10 mM 2-mercaptoethanol (pH 7.5) containing ^{65}Zn (1 $\mu\text{Ci}/\text{ml}$). As a control, ^{65}Zn binding in the absence of these competing ions was measured. After 15 min, the nitrocellulose dots were washed twice for 8 min each in buffer (10 mM Tris/HCl, 10 mM 2-mercaptoethanol, pH 7.5) and radioactivity was measured by liquid scintillation counting.

Assay of metal binding *in vivo*

A ^{65}Zn solution (5 μCi of ^{65}Zn ; 25 μM ZnSO_4 in 0.9% NaCl) was introduced into a 10 cm ligated intestinal loop of an anaesthetized rat (Hempe and Cousins, 1991). To study the

binding affinities of the other metals to CRIP *in vivo*, 5 μ Ci of ^{64}Cu (25 μM CuSO_4 , pH 4.0) and 5 μ Ci of ^{109}Cd (0.25 μM CdCl_2 , pH 7.5) were separately injected into ligated duodenal loops. High-resolution gel-filtration chromatography (Superdex 75 Hiload column) was used to measure metal binding to CRIP. Fractions containing CRIP were identified by elution of purified CRIP, the *in vitro* ^{65}Zn -binding assay and a cadmium-binding assay to distinguish CRIP from MT (Hempe and Cousins, 1991).

Comparative binding affinities of CRIP and MT

MT (30 μM) and CRIP (30 μM) were placed in two different dialysis bags (Spectropor-3 membrane) and incubated in 250 ml of Tris buffer (10 mM Tris/HCl, 154 mM NaCl, 1 mM MnCl_2 , pH 7.5) with 1.0 mM DTT and 1 $\mu\text{Ci/ml}$ ^{65}Zn (carrier-free). Another dialysis bag without protein was used as a control. After 4 h the contents were passed through 10 cm columns of Sephadex G-25 to remove unbound ^{65}Zn , in order to compare the relative binding of ^{65}Zn to MT and CRIP. Rat MT was prepared as described by Grider et al. (1989).

RESULTS

Initial separation of CRIP from other low- M_r proteins in the cytosol fraction used low-resolution gel-filtration chromatography. To identify the elution characteristics of CRIP from the Sephadex G-75 column, the first batches of cytosol were from the mucosa of ligated intestinal loops where ^{65}Zn had been introduced to label the zinc-binding proteins *in vivo*. As shown in Figure 1(a), the major ^{65}Zn -binding peak in the low- M_r range has a K_{av} of 0.77. Previous experience has shown that these fractions will have a high abundance of CRIP and relatively little MT. For comparison (Figure 1b), when cytosol from rats given zinc injections to induce MT was separated in an identical fashion, substantial ^{65}Zn was found bound to MT (K_{av} of 0.70).

Actual purification steps used *in vitro* ^{65}Zn blotting as a means to establish the presence of CRIP in the chromatographic fractions. A ^{65}Zn blot of fractions from Sephadex G-75 chromatography of intestinal cytosol from non-zinc-treated rats is shown in Figure 2. Maximum binding intensity is found at an elution volume of 340–380 ml (K_{av} of 0.77).

High-resolution f.p.l.c. using a Superdex 75 Hiload column was the next purification step. Comparison of absorbance at 280 nm with the intensity of the zinc blots suggests that Superdex 75 Hiload chromatography yielded very impure CRIP (Figure 3). PAGE and microsequencing of the CRIP-containing fractions from the Superdex column confirmed that a considerable number of contaminating proteins co-eluted at this step.

After Superdex chromatography, differing approaches to further purification were used. One approach was continuous elution PAGE (total time was 7 h). As shown in Figure 4(a), several PAGE fractions had ^{65}Zn -binding activity, based on the ^{65}Zn blot assay. Figure 4(b) shows analytical SDS/PAGE of sequential continuous elution fractions showing maximum ^{65}Zn binding activity. A fraction eluting just prior to CRIP was identified as ubiquitin by microsequencing. Careful selection of CRIP-rich fractions and maintenance of low temperatures limit contamination with ubiquitin.

Figure 5(a) shows analytical SDS/PAGE of the purification sequence from intestinal cytosol to CRIP using continuous elution PAGE as the final step. The CRIP preparation migrated as a single band near the 6000- M_r standard. Microsequencing confirmed purity. The sequence for 20 cycles was PKCPKCDKEVYFAERVTSLG, which agrees completely with earlier results (Hempe and Cousins, 1991). Yield is somewhat difficult to estimate, but approx. 200 μg of CRIP was recovered

per g of cytosol protein (i.e. 0.02 % of total cytosolic protein). This would give a tissue concentration for CRIP of approx. 15–20 $\mu\text{g/g}$ of tissue. These values agree favourably with the CRIP mRNA abundance of 0.3 % of total cellular RNA (Birkenmeier and Gordon, 1986).

Figure 1 Absorbance and ^{65}Zn profile of fractions from Sephadex G-75 gel-filtration chromatography

^{65}Zn -binding components of intestinal cytosol from rats were separated on a Sephadex G-75 gel-filtration column (2.5 cm $\times$ 90 cm; 4 ml fractions). The absorbance at 280 nm (solid line) and the recovery of radioactivity (bars) were used to identify the high- and low- M_r regions. (a) Cytosol from normal rats. At the low- M_r region, the radioactive peak eluted at a V_e/V_0 of approx. 2.1–2.4 (K_{av} 0.77). (b) Cytosol from rats where MT was induced by zinc administration. At the low- M_r region, the radioactive peak eluted at a V_e/V_0 of approx. 1.9–2.1 (K_{av} 0.70). This is characteristic elution behaviour for MT by this method of chromatography.

Figure 2 *In vitro* ^{65}Zn metal blot of Sephadex G-75 chromatography fractions

Sephadex G-75 gel filtration fractions of unlabelled cytosol (Figure 1a; between elution volumes of 200 and 440 ml; V_0 160 ml) were blotted on a nitrocellulose membrane at 200 $\mu\text{l/well}$. The membrane was incubated with ^{65}Zn . Major ^{65}Zn -binding fractions were between 340 and 380 ml (V_e/V_0 2.1–2.4), as visualized by autoradiography.

Figure 3 Absorbance and ^{65}Zn metal blot of fractions from the Superdex 75 HiLoad column

(a) Elution profile (A_{280}) of CRIP samples using Superdex 75 HiLoad high-performance gel-filtration chromatography. Isocratic elution (1.0 ml fractions) was carried out at 0.5 ml/min. (b) ^{65}Zn -blot assay of fractions vacuum-blotted on a nitrocellulose membrane (as described in Figure 2) shows that CRIP elutes between fractions 60 and 62 (16–17 ml).

Figure 4 ^{65}Zn metal blot assay and analytical SDS/PAGE of fractions from continuous elution electrophoresis

(a) Fractions (2.0 ml) from continuous elution electrophoresis were blotted on nitrocellulose and assayed by the zinc blot assay, as described in Figure 2. CRIP was detected in the zinc-binding fractions. (b) Sequential protein-containing fractions around the ^{65}Zn -binding fractions were also analysed by analytical SDS/PAGE, and transferred on to PVDF membranes. The fraction eluting prior to CRIP was identified as ubiquitin by microsequencing.

An alternative method of preparing CRIP uses CM-Sephadex chromatography. Using 10 mM potassium phosphate buffer with a 0–350 mM KCl gradient, CRIP eluted from the column at approx. 150 mM KCl. Most of the zinc eluted with the CRIP at 150 mM KCl (results not shown). As shown in Figure 5(b), a single band was produced with virtually identical migration

Figure 5 SDS/PAGE of the two different purification schemes for CRIP from intestinal cytosol to pure protein

(a) Samples from intestinal cytosol to continuous elution electrophoresis were analysed by SDS/PAGE and stained with silver stain. Pure CRIP elutes at approx. M_r 6000. Lanes: 1, M_r standards; 2, rat intestinal cytosol; 3, fraction from low-resolution chromatography (Sephadex G-75); 4, fraction from high-resolution chromatography; 5, pure CRIP after continuous elution electrophoresis. (b) SDS/PAGE of the purification sequence using CM-Sephadex C-25 chromatography as the final step. Lanes: 1, M_r standards; 2, rat intestinal cytosol; 3, sample from Sephadex G-75 chromatography; 4, CRIP after CM-Sephadex cation-exchange chromatography; 5, pure CRIP after Superdex 75 HiLoad chromatography.

Figure 6 Mass spectrum of CRIP obtained by continuous elution electrophoresis

Mass is shown from M_r 7000 to 9500. The peak derived has an M_r of 8392 which is consistent with the calculated M_r minus methionine.

characteristics to those of CRIP prepared by continuous elution PAGE. Overloading of CM-Sephadex C-25 can produce a slightly impure CRIP that can be purified to homogeneity using Superdex 75 HiLoad chromatography. Microsequencing verified the identity of the protein as CRIP of comparable purity.

The zinc content of CRIP prepared using continuous elution PAGE and CM-Sephadex chromatography yielded stoichiometries of 0.3 mol of Zn/mol of CRIP and 0.6 mol of Zn/mol of CRIP respectively. Supplementation of buffers with 10 μM Zn(II) yielded 2 mol of Zn/mol of CRIP. Because CRIP obtained by continuous PAGE was lowest in zinc content, it was used for the characterization steps that followed. The absorption spectrum of purified CRIP is consistent with that expected for a protein with a number of constituent aromatic amino acids (results not

Figure 7 Effect of pH on the binding affinity of CRIP for ⁶⁵Zn

Purified CRIP (40 μ l; 0.4 mg/ml) from the continuous gel electrophoresis step was incubated with 0.1 μ Ci of ⁶⁵Zn in 160 μ l of 50 mM Tris/acetate buffer (pH 3.5–5.5) or 160 μ l of 50 mM Hepes buffer (pH 6.5–9.5) for 30 min on ice. The solution was diluted with 4 vol. of 50 mM Hepes, pH 7.5, and centrifuged with Centricon-3 tubes for 20 min at 3600 rev./min (r_{av} , 6.5 cm). The ⁶⁵Zn bound as a percentage of the total ⁶⁵Zn in the incubation buffer was measured by subtracting the radioactivity of the filtrate after filtration from the radioactivity of the mixture before ultrafiltration. Each point is the mean $\pm$ S.E.M. ($n = 3$).

Figure 8 Comparative *in vitro* metal binding of purified CRIP

Purified CRIP (5, 10, 15 and 20 μ g) from the continuous elution electrophoresis step was vacuum-blotted on nitrocellulose strips. These were incubated with 5 μ Ci of ⁶⁵Zn, ⁶⁷Cu, ¹⁰⁹Cd or ⁵⁹Fe in 50 ml of 10 mM Tris/HCl, pH 7.5, for 15 min after 2 h of prewashing (10 mM Tris/HCl, 1 mM MnCl₂, 10 mM 2-mercaptoethanol, pH 7.5). The strips were washed three times (10 min each) and were visualized by autoradiography.

shown). Addition of zinc did not alter the spectrum. Mass spectral analysis showed an M_r of 8392 (Figure 6), which is consistent with that calculated from the CRIP sequence (M_r 8550). Presumably the difference is due to the cleavage of methionine from the protein.

The effect of pH on the zinc-binding ability of CRIP was studied between pH 3.5 and 9.5. Correction was made for membrane retention of radioisotope and recovery of protein. There was a plateau of ⁶⁵Zn-binding activity between pH 5 and 8 at about 25% bound (Figure 7). Binding decreased below pH 4.5 to about 8%, and increased above pH 8.5 to 40%.

In vitro binding assays showed that ⁶⁵Zn, ⁶⁷Cu and ¹⁰⁹Cd bound to purified CRIP immobilized on nitrocellulose, but ⁵⁹Fe did not (Figure 8). A concentration-related increase was observed for each metal bound. The ability of copper and cadmium, in

Figure 9 *In vitro* competition binding assay

Purified CRIP (20 μ g) from continuous elution electrophoresis was vacuum-blotted as dots on to nitrocellulose. The dots were incubated individually in 6-well plates with buffer containing Zn(II) (■), Cu(II) (●) or Cd(II) (▲) (2 ml; 10⁻⁶–10⁻³ M metal, pH 7.0) with 10 μ Ci of ⁶⁵Zn for 20 min at room temperature. The dots were washed and the competition ⁶⁵Zn binding of CRIP was compared with the binding without competing ions in the incubation buffer, which was calculated to 100%. Each point is the mean $\pm$ S.E.M. ($n = 3$).

increasing concentrations (10⁻⁶–10⁻³ M), to compete with ⁶⁵Zn for binding to CRIP was examined using CRIP immobilized on nitrocellulose dots. As shown in Figure 9, cadmium and zinc showed comparable competition except at the lowest concentration tested (10⁻⁶ M).

In vivo binding studies with ligated loops of rat intestine showed that nearly 10-fold more ⁶⁵Zn than ⁶⁴Cu or ¹⁰⁹Cd found in the cytosol after absorption was bound to CRIP 15 min after the radioisotopes were placed in the intestinal lumen. On a percentage basis, ⁶⁵Zn binding amounted to 33% associated with the CRIP fraction. ⁵⁹Fe did not bind to CRIP under these conditions (results not shown).

Purified CRIP provides the opportunity to examine questions with physiological implications. To examine the relative affinities of CRIP and MT for zinc, equimolar amounts of each protein were placed in separate dialysis membranes and dialysed with buffer containing ⁶⁵Zn. Subsequently, each protein solution was passed through a small gel-filtration column to obtain protein-bound ⁶⁵Zn. Under the conditions used, MT bound 4600 c.p.m. while CRIP bound 400 c.p.m. (results not shown). This reflects a relative zinc-binding affinity ratio of MT/CRIP of 12:1 under these conditions.

DISCUSSION

The purification schemes described in this paper are the first to yield homogeneous preparations of CRIP from the intestine. The methods require attention to inhibition of proteolysis. Since intestinal mucosa rather than intestinal cells was the source of CRIP, a variety of protease inhibitors were used. Even with these measures, if care was not exercised to avoid sample heating and if special attention was not given to stringent choice of fractions to pool for subsequent steps, the end-product was contaminated with ubiquitin. We have confirmed the identity of this co-migrating contaminating protein by peptide sequencing. Further studies will be necessary to confirm the degradation pathway for CRIP, but these results suggest that it is via a ubiquitin-dependent mechanism.

Repeated attempts to obtain CRIP with zinc in amounts

expected of a double zinc finger motif proved unsuccessful. This was circumvented by direct addition of Zn(II) to buffers used for chromatography. Preparation of CRIP from rats injected with zinc did not improve the situation, since this produces a massive increase in MT abundance. We cannot rule out the possibility that, *in situ*, CRIP has a stoichiometry of less than 2 mol of Zn/mol, and that its physiological function relates to zinc loss or accretion (Chesters, 1992).

The electrophoretic data presented here, as well as that provided earlier (Hempe and Cousins, 1991), would suggest that CRIP has an M_r of not more than 6000. The presence of CRIP in this electrophoretic band has been established by micro-sequencing. M_r determinations from the mass spectrum using the pure protein indicate that the protein has an M_r of 8392. The apparent discrepancy may be due to metal loss associated with purification and electrophoresis. Loss of metal-related tertiary structure would explain the difference between electrophoretic mobility and true M_r . The loss of structure may also explain in part why CRIP elutes much later than MT when separated by gel-filtration size-exclusion chromatography. The Stokes radius of MT is such that elution by size makes it appear much larger than its apparent M_r of approx. 6500 (Kagi and Kojima, 1987). This also contributes to the lower K_{av} of CRIP (0.77) compared with MT (0.70).

For years a number of laboratories, including ours, have been studying the changes in intestinal zinc transport (transcellular movement) as correlated to low- M_r zinc-binding fractions from gel-filtration chromatography (reviewed in Cousins, 1985). Without question, one of the proteins found in these fractions was MT. This has been established through amino acid analysis (Richards and Cousins, 1977), MT mRNA translation (Menard et al., 1981) and Northern hybridization analyses (Cousins and Lee-Ambrose, 1992). This gene is clearly up-regulated in response to increases in the dietary zinc supply.

Our more recent experiments (Hempe and Cousins, 1991, 1992; Levenson et al., 1993) and the data presented here clearly show that CRIP is also a constituent of these low- M_r fractions from gel-filtration chromatography. Furthermore, the present results show that past data on the relationship between MT and zinc transport, which relied only on gel-filtration elution data for protein identification, must be viewed with caution. These fractions represent a composite of many proteins, including at least two zinc metalloproteins, i.e., CRIP and MT. Our previous and present data suggest that CRIP is constitutively expressed in adult rats (Levenson et al., 1993) and, as shown here, is more abundant than MT in the small intestine when the dietary zinc supply is not above the adequate range.

Since the small intestine and colon are major sites of CRIP expression, a role for the protein in nutrient absorption is attractive, and this actually was postulated by Birkenmeier and Gordon (1986). This speculation is enhanced by data showing that, once introduced into the intestinal lumen, zinc binds to CRIP when identified from the cytosol of mucosal scrapings (Hempe and Cousins, 1991, 1992). In contrast, CRIP does not bind ^{65}Zn when the isotope is added directly to a homogenate or cytosol. This suggests that labelling of CRIP *in vivo* is the result of a cellular process rather than only a metal exchange at a labile binding site or metal binding to fill unoccupied binding sites. Our data on competitive metal binding, pH-dependence for binding and zinc binding affinity are consistent with a zinc-related function. However, CRIP mRNA is also abundant in spleen, which argues against a role unique to the intestine. Furthermore,

structural data and the stoichiometry of zinc binding reported here are consistent with a role in DNA or RNA binding via the dual zinc finger LIM motif (Freyd et al., 1990; Boehm et al., 1991; Wang et al., 1992), in protein-protein interactions (Sadler et al., 1992) or in protein translocation (Tsonis et al., 1988). Our calculation for the abundance of CRIP in rat intestine suggests that it is much more abundant than well characterized transcription factors. All potential physiological roles need to be placed within the context of developmental regulation of CRIP, particularly in intestinal cells (Birkenmeier and Gordon, 1986; Levenson et al., 1993).

The LIM motif most likely provide CRIP with the zinc finger binding domains which may interact with zinc in various cellular compartments. As mentioned here, MT is capable of competing more favourably for available zinc in solution. This is of interest, since we have shown earlier that, as MT expression decreases in neonatal small intestine, CRIP expression increases (Levenson et al., 1993). The zinc-binding properties of MT have been suggested as serving a regulatory role in zinc finger-containing transcription factors, e.g. Sp-1, via zinc removal (Zeng et al., 1991). By analogy, a zinc-dependent function for CRIP in developing intestine may require that MT expression is reduced.

In summary, the purification procedures described in this paper have established methods to obtain useful quantities of CRIP from the small intestine. Using these methods, sufficient purified CRIP can be produced for use in experiments designed to study the physiological role of this protein in intestinal cells and perhaps in other cells.

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