Oral Absorption of Peptides Through the Cobalamin (Vitamin B12) Pathway in the Rat Intestine

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Purpose. This study was aimed at examining the extent and mechanism of uptake of cobalamin (Cbl)-conjugated peptides in vitro and in vivo.
Methods. To enable acquisition of quantitative absorption data of Cbl-peptides, metabolically stable octapeptides (DP3), with (Cbl-Hex-DP3) or without a hexyl spacer (Cbl-DP3), were coupled to Cbl and radiolabeled. For comparison, LHRH coupled to Cbl was used as metabolically susceptible peptide. Biological recognition of Cbl-peptides was studied in the physiological order: binding by Intrinsic Factor (IF), recognition and transport of the IF-complexes by IF-Cbl receptors (IFCR) on Caco-2 monolayers and oral absorption of the Cbl-conjugates in the rat.
Results. All Cbl-peptides bound to IF and the IF-complexes were recognized by IFCR receptors on Caco-2 monolayers. Binding was saturable and could be inhibited by a 20-fold excess of IF-Cbl, but not of Non-intrinsic Factor (NIF)-Cbl. Oral administration of these ligands to rats resulted in absorption of 53%, 45%, 42%, and 23% of the applied radioactivity for Cbl, Cbl-LHRH, Cbl-Hex-DP3, and Cbl-DP3, respectively. Simultaneous administration of a $>10^5$-fold excess of unlabeled Cbl reduced uptake of all compounds to $<4\%$. Tissue distribution and elimination of the metabolically stable Cbl-conjugates were comparable to Cbl.
Conclusions. The endogenous Cbl uptake pathway can be exploited for oral peptide delivery as indicated by the specific and high (40–45%) uptake of metabolically stable Cbl-coupled octapeptides.

KEY WORDS: cobalamin (vitamin B12); in vitro-in vivo study; Cbl-peptide conjugate; oral absorption.

INTRODUCTION

The increasing availability of therapeutic peptides and proteins would be well served with the development of convenient methods of administering these molecules to the patient. Polypeptides are susceptible to proteolytic degradation and do not diffuse across biological membrane barriers, and are therefore commonly administered by injection. To avoid the encumbrance of parenteral application, most epithelial surfaces are being evaluated as alternative routes of peptide administration (1–3). Oral administration of drugs is the most convenient, economical, and acceptable route and also has the possibility to achieve sustained plasma levels of the peptide. Up until now, approaches for oral administration of peptides have involved the use of complex emulsion systems (4), enzyme inhibitors (5), detergent-like absorption enhancers (6), colonic delivery (7), or via the intestinal peptide and bile acid transporters (8,9) but only limited success has been achieved to date.

Only a few oligopeptides *per se* meet the physicochemical and structural characteristics that allow for passive or active absorption from the intestine (10,11). For the vast majority of larger polypeptides, oral absorption is limited by their susceptibility to proteolytic degradation and, often more significantly, by their inefficient transport across cellular barriers (4,11). To some extent, both of these barriers can be circumvented by using peptidase inhibitors and detergent-like compounds that facilitate absorption across membranes, although there may be significant safety concerns with their routine use (6).

To avoid the use of enhancers and achieve selective peptide absorption we have attempted to utilize the endogenous intestinal uptake pathway for cobalamin (Cbl) absorption. Cobalamin is actively taken up in the small intestine by receptor-mediated transcytosis. After binding to intrinsic factor (IF), a 48K gastric protein, the IF-Cbl complex is recognized and internalized by IF receptors (IFCR) on the surface of the ileal epithelium. Cobalamin is subsequently transported to the basolateral membrane and, two to three hours after internalization, it appears in the circulation complexed with Transcobalamin II (TCII) (12–14).

Radiolabelling of peptides facilitates mechanistic and quantitative studies on peptide absorption but their proteolytic degradation may result in absorbable iodinated degradation products that obscure data interpretation considerably. In the present investigation, the mechanism and extent of absorption of Cbl-coupled peptides has been studied both *in vitro* and *in vivo*. To enable a clear interpretation of the data and to avoid any misinterpretation by metabolism or pharmacological effects, a pharmacologically inactive octapeptide was selected for the current "proof of concept" study that is reported to possess excellent resistance against proteolytic degradation (15).

Given the fact that the GI tract is designed to break down peptides, the concept of oral protein delivery seems ill-defined; and even the early phases of the cobalamin delivery pathway such as recognition by IF are not without controversy. Early studies suggested that, with the exception of substitutions at the Co-β position (cyanide in native Cbl), substitutions of the Cbl molecule compromise its recognition by IF (16,17), although some other groups have reported substantially better IF binding by VB12 conjugates (18)

Therefore, a consecutive series of experiments was designed to study molecular interactions and recognition of the Cbl-peptides with its natural ligands in the physiological order: (i) binding of the Cbl-peptides to IF; (ii), binding of IF-Cbl-peptides to specific cellular receptors (IFCR) on mature Caco-2 monolayers, (iii) transcytosis of the Cbl-peptide across an endothelial barrier (Caco-2 cells) and (iv), confirmation of the *in vitro* results *in vivo* using the rat as a model.

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ABBREVIATIONS: Cbl, cobalamin (Vitamin B12, VB12); CD-29, monoclonal anti-Cbl antibody; IF, intrinsic factor; IFCR, IF-Cbl receptor; NIF, non-intrinsic factor; TCII, transcobalamin II.

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MATERIALS AND METHODS

Reagents

All cell culture media were from Gibco BRL. [^{57}Co]-Cbl was purchased from Amersham. Cobalamin, a monoclonal anti-VB12 antibody CD-29 and IF and NIF from porcine gastric mucosa were purchased from Sigma. The polyclonal rabbit anti-LHRH antibody was from Biotech Australia, Roseville, Australia. Cbl-LHRH was prepared as described previously (19). DP3 (Glu-Ala-Ser-Ala-Ser-Tyr-Ser-Ala) was synthesized from D amino acids (Hoffmann-La Roche Ltd., Basle) according to Pappenheimer *et al.* (15). Conjugates of DP3 and the "e" isomer of the monocarboxylic acid of Cbl (20) were prepared either by directly coupling to the N-terminus (Cbl-DP3; MW 2123) or via a hexyl spacer (Cbl-Hex-DP3; MW 2236) using EDAC (1-ethyl-3-((3-dimethylaminopropyl)carbodiimide) (19). All other reagents were from Sigma and Fluka.

Cell Culture

Human colon carcinoma Caco-2 cells (passage numbers 100-113, ECACC, England) were seeded at a density of 70,000 cells/cm^2 on Costar collagen-coated 12 mm Transwells (0.45 μm) and cultured at 37°C in a humidified 5% CO_2 atmosphere. After the first 48h, media (DMEM (Dulbecco's Modified Eagle Medium) with 10% Fetal Calf Serum (Gibco), 1% nonessential amino acids, 100 U/ml penicillin and 100 μg streptomycin) were changed every two days in both the apical (0.5 ml) and the basolateral (1.5 ml) chambers. Experiments were performed with cells grown for 21–25 days after functionally tight monolayers were formed as assessed by transepithelial electrical resistance and [^{14}C]-mannitol permeability. DMEM containing 1% nonessential amino acids, 100 U/ml penicillin and 100 μg streptomycin and 0.1% BSA was used for binding and transcytosis studies (transport buffer).

Binding of Cbl-DP3, Cbl-Hex-DP3 and of Cbl-LHRH to Cbl-Binding Proteins

MaxiSorp-breakapart microtiter plates (Nunc) were coated overnight at 4°C with 100 μl avidin (Fluka, 5 μg/ml in 0.1 M bicarbonate buffer, pH 9.6). Remaining binding sites were blocked for 2 h at room temperature with 150 μl of 1% bovine serum albumin in 0.2 M Tris buffer, pH 7.3 and 100 μl of biotinylated intrinsic (IF) or non-intrinsic factor (NIF) (0.15 U/ml) were added for 2 h at room temperature (Alsenz *et al.*, manuscript in preparation). Mixtures of a constant concentration of 0.5 ng/ml [^{57}Co]-Cbl (3.7×10^{-10} M) (Amersham) and serial dilutions of cobinamide, cobalamin (Cbl)(Sigma), Cbl-LHRH, Cbl-DP3 or Cbl-Hex-DP3 were added. After an overnight incubation at 4°C, wells were washed and counted for [^{57}Co]-radioactivity.

Labeling of Cbl-Peptides with Iodine

Cbl-conjugates were labeled with [^{125}I]odine (Amersham) using chloramine T as oxidant. Reaction was stopped with ascorbic acid and the peptide was separated from free [^{125}I] by using a Supelco RP-18 minicolumn. Any remaining free [^{125}I] was further removed by incubation of the Cbl-peptides with an excess of CD-29 (the antibody binds to the Cbl moiety) for 1 h at RT followed by centrifugation through a Centricon-10 concentrator (Amicon). The retentate, containing [^{125}I]-peptide bound to CD-29 was washed three times with PBS to remove free [^{125}I]. Cbl-peptides were subsequently eluted from CD-29 by washing of the retentate four times with 100 μl 1N HCl. After neutralization of the filtrate with an equal volume of 1N NaOH and addition of bovine serum albumin to a final concentration of 0.1%, samples were stored frozen in aliquots until use.

Association of IF-[^{57}Co]-Cbl, IF-Cbl-[^{125}I]-DP3, IF-Cbl-Hex-[^{125}I]-DP3 or IF-Cbl-[^{125}I]-LHRH with Caco-2 Monolayers and Transcytosis of Complexes

Confluent Caco-2 monolayers were cultured as described under 'cell culture.' Cells were placed on ice and washed three times with prechilled transport buffer (see above). Prechilled solutions of the various labeled IF- or NIF-complexes (100 fMol/filter) were added to the apical side of the cells in the presence or absence of a 20-fold excess of unlabelled IF-Cbl or NIF-Cbl complexes. After incubation for 2 h at 4°C, samples were removed, the cells were washed three times with DMEM/0.1% BSA and measured for radioactivity. Complexes were prepared by incubating IF or NIF with a 20% molar excess of Cbl, [^{57}Co]-Cbl, Cbl-[^{125}I]-DP3, Cbl-Hex-[^{125}I]-DP3 or Cbl-[^{125}I]-LHRH in DMEM/0.1% BSA for 1 h at RT. Samples were loaded onto Centricon-10 concentrators and complexes (concentrate) were separated from unbound ligands (filtrate) by centrifugation and subsequent washing the concentrate 3 times with 0.3 ml DMEM (5000 × g, 10 min, 4°C). For transcytosis studies, 100 fMol of [^{57}Co]-Cbl or Cbl-[^{125}I]-peptide alone or complexed with IF or NIF in DMEM/MEM/Pen-Strep/10%FCS was added to each well for 20 h at 37°C. Medium was removed and radioactivity in the basolateral medium was determined. The cells were washed twice with 0.5 ml 50 mM Tris/2.5 mM Ca^{2+}/0.1% BSA, pH 7.4 and surface-bound radioactivity was separated from internalized by (i) washing the cells twice rapidly with 0.5 ml glycine-buffer, pH 3 at 4°C as suggested by Dix *et al.* (19) and (ii) once with 0.5 ml of trypsin-EDTA (Gibco) for 10 min at 37°C; the latter was found to remove surface-bound radioactivity much more efficiently than glycine buffer alone. Cells detached by the washing procedure were collected by centrifugation and counted together with the other cells (internalized radioactivity). Glycine and Trypsin/EDTA washes were combined and radioactivity was determined.

In Vivo Studies with [^{57}Co]-Cbl, Cbl-[^{125}I]-LHRH, Cbl-Hex-[^{125}I]-DP3, and Cbl-[^{125}I]-DP3 in the Rat

The oral absorption of [^{57}Co]-Cbl, Cbl-[^{125}I]-LHRH, [^{125}I]-DP3, Cbl-[^{125}I]-Hex-DP3 and Cbl-[^{125}I]-DP3 was studied in conscious male Wistar rats after an overnight fast. Samples (1 pMol) were dissolved in 1 ml buffer (PBS/0.1% BSA) with or without a $>10^5$-fold excess of Cbl and administered into the stomach of rats by gavage. At the end of the experiment, rats were sacrificed, the intestine was washed thoroughly (>500 ml) with PBS/0.1% BSA to remove unabsorbed ligands and radioactivity in the washing fluid, in urine and in tissue (liver, kidney, spleen, heart, lung, stomach, skin, muscle, testis, brain, plasma, colon, caecum and washed small intestine) was measured. In case of muscle and skin, two to three 3–4 g samples

were counted and total radioactivity was calculated assuming that 60% and 20% of the total body weight represent muscles and skin, respectively. Recoveries of radioactivity were generally >85% of the administered dosages. Peptide absorption is expressed as % of administered dosage recovered from tissues and urine. Tissue distribution is expressed as percentage of absorbed dosage. Since the maximal uptake of [^{57}Co]-Cbl in rats was 9–13 pMol per dosage (data not shown), oral administration of Cbl-conjugates was limited to 1 pMol to avoid an overloading of the Cbl-uptake capacity.

For i.v. studies, 1 pMol of each peptide was injected into the vein of cannulated conscious rats. A cannula was implanted into the jugular vein two days before the experiment. At the end of the experiment, rats were sacrificed and tissue distribution determined as described above. Tissue distribution is expressed as percentage of administered dosage.

All animal experiments adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Integrity of Cbl-Hex-[^{125}I]-DP3 Recovered from Rat Kidney After Oral Administration to Rats

Cbl-Hex-[^{125}I]-DP3 was administered orally to rats as described above. After 24 h, the kidneys containing 10–13% of the administered radioactivity were collected and separately homogenized in 1 ml PBS for 2 min at room temperature using a Polytron. The homogenates were centrifuged (Biofuge 15, Heraeus) (10 min, 19,000 g, room temperature) and the supernatant was collected. The tissue pellet was extracted further with 2 × 0.5 ml PBS and with 2 × 0.5 ml water/1% Triton X-100/10 mM EDTA. The combined supernatants, which contained approximately 75% of the total radioactivity, were ultracentrifuged at 40,′00 × g for 45 min at 4°C. Larger molecules were removed by passing the supernatant sequentially through Centricon-100 and Centricon-10 concentrators. The Centricon-10 filtrate was mixed with 20 μl of an anti-cobalamin antibody (CD-29, undiluted) (Sigma), incubated for 4 h at 4°C and passed through another Centricon-10 concentrator. The Centricon-10 concentrate containing >70% of the extracted radioactivity (= kidney extract; CD-29-or TCII-bound Cbl-peptide that is retained by the Centricon-10 filter) was analyzed by SDS-PAGE (PhastGel High Density) and by isoelectric focusing (IEF) (PhastGel IEF pH 3–9) using a Phastsystem (Pharmacia) for the separation and a Phosphor screen (Molecular Dynamics) for the detection of radioactivity.

RESULTS

Binding of Cbl and Cbl-Conjugates to Cbl-Binding Proteins

Affinity of Cbl-conjugated peptides to IF and NIF was measured by a competition experiment (Fig. 1). Microtiterplates coated with IF or NIF were incubated with a mixture of [^{57}Co]-Cbl and serial dilutions of Cbl, Cbl-LHRH, Cbl-DP3, Cbl-Hex-DP3, and cobinamide. In contrast to NIF, IF has a very low affinity for the Cbl analogue cobinamide. Cbl-LHRH bound to IF with identical affinity as compared to Cbl, whereas the affinity of the Cbl-DP3 conjugate was 8-fold lower. Insertion of a hexyl spacer between the Cbl and the DP3 moiety improved recognition by IF by a factor of about 5, while cobinamide had IC_{50} 4 orders of magnitude higher. In contrast to IF, NIF had about the same affinity for all tested conjugates.

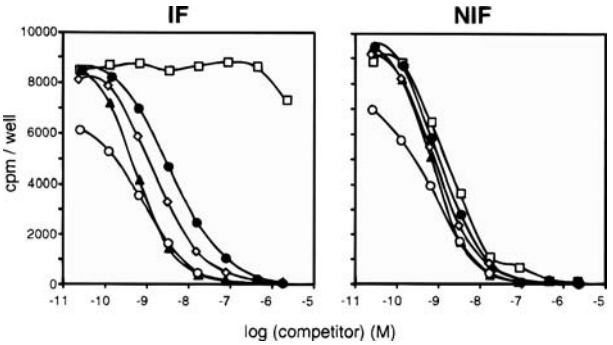

Fig. 1. Binding of Cbl-DP3, Cbl-Hex-DP3, and of Cbl-LHRH to Intrinsic Factor (IF) and non-intrinsic factor (NIF). Microtiter plates coated with IF or NIF were incubated with a mixture of [^{57}Co]-Cbl (3.7×10^{-10} M) and serial dilutions of cobinamide (□), cobalamin (Cbl) (▲), Cbl-LHRH (○), Cbl-DP3 (●) or Cbl-Hex-DP3 (◇). After an overnight incubation at 4°C, wells were washed and counted for bound [^{57}Co]-radioactivity.

Recognition of IF-Cbl-Peptide Complexes by Cellular IF-Cbl-Receptors

In order to study recognition of complexes of Cbl-peptide conjugates with IF by high affinity binding sites on polarized Caco-2 cells, a series of binding experiments at 4°C was performed. Radiolabeled ligands were pre-complexed to IF or NIF (as negative control), and incubated in the absence or presence of a 20-fold excess of unlabeled IF-Cbl or NIF-Cbl. Cellular association of IF-complexes were 5–10-fold higher than the corresponding NIF-Cbl-peptide complexes (Fig. 2). Co-incubation with an excess of unlabeled IF-Cbl blocked association of IF-Cbl-peptide 5–9-fold, while NIF-Cbl had no effect on surface association. Recognition of all IF-Cbl-peptide complexes by IFCR receptors on Caco-2 cells was identical to IF-Cbl complexes.

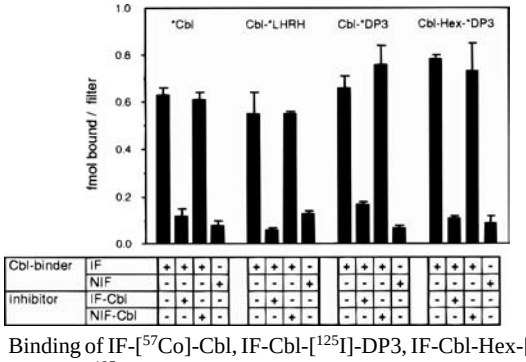

Fig. 2. Binding of IF-[^{57}Co]-Cbl, IF-Cbl-[^{125}I]-DP3, IF-Cbl-Hex-[^{125}I]-DP3, or IF-Cbl-[^{125}I]-LHRH to polarized monolayers of Caco-2 cells and inhibition of binding by IF-Cbl and NIF-Cbl. Prechilled solutions (200 pM) of [^{57}Co]-Cbl, Cbl-[^{125}I]-LHRH, Cbl-Hex-[^{125}I]-DP3 or Cbl-[^{125}I]-DP3, pre-complexed to either IF or NIF, were added to the apical surface of filter-grown Caco-2 cells (0.5 ml/well). Specificity of binding was assessed in the presence of a 20-fold molar excess of IF-Cbl or NIF-Cbl. Cells were incubated for 2 h at 4°C, washed and measured for bound radioactivity. Values represent averages of triplicate determinations.

Transcytosis of IF-Cbl-Peptide Complexes Across Caco-2 Cell Monolayers

Receptor-mediated transcytosis of [^{57}Co]-Cbl and of Cbl-[^{125}I]-peptide conjugates was studied in vitro using Caco-2 cell monolayers. Cbl or Cbl-conjugates alone (buffer) or pre-complexed with either IF or NIF were added to the apical side of Caco-2 cell monolayers and incubated for 20 h at 37°C. Subsequently, transcytosed, surface-bound and internalized radioactivity were determined.

The results presented in Fig. 3 suggest that, based on the transcytosed radioactivity, the transcytosis of Cbl-LHRH and of Cbl-Hex-DP3 complexes in the presence of IF was in about the same range as Cbl (3–5 fMol/filter). In contrast, significantly less Cbl-DP3 was transported across Caco-2 cell monolayers ($\leq$1.5 fMol/filter), although the surface-bound and internalized radioactivity was comparable to the other conjugates tested. Binding, internalization and transcytosis of the Cbl-peptide conjugates was specific, since complexes with NIF were not transported or taken up by the cells.

Transcytosed Cbl and Cbl-conjugates are anticipated to be present in the basolateral compartment complexed to TCII (21). Basolateral media were analyzed for the amount and integrity of Cbl-conjugates by centrifugation through Centricon-30 concentrators. The latter contain TCII-Cbl and TCII-Cbl-peptide conjugates in the retentate and free peptides or degradation products in the filtrate. Centricon-30 analysis showed that 96%, 77% and 85% of [^{57}Co]-Cbl, Cbl-[^{125}I]-DP3 and Cbl-Hex-[^{125}I]-DP3, respectively, were present in the retentate (Table 1). In contrast, >50% of the radioactivity of Cbl-[^{125}I]-LHRH was in the filtrate. Incubation of the retentates for 12 h at 37°C with a >10,000-fold excess of cold Cbl released almost all the bound radioactivity which can subsequently be recovered in the filtrate after Centricon-30 centrifugation. In case of Cbl-[^{125}I]-LHRH, subsequent incubation of the filtrate with a polyclonal anti-LHRH antibody and filtration through Centricon-30 concentrator did not retain the radioactivity in the retentate. In contrast, when Cbl-[^{125}I]-LHRH was premixed with basolateral medium, 91% of the radioactivity bound to the anti-LHRH antibody under the same experimental conditions (Table 1). Therefore, most of the transcytosed Cbl-LHRH conjugate seems to represent degraded material.

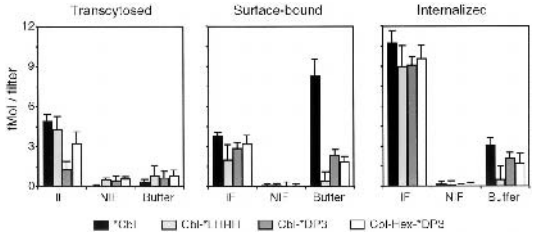

Fig. 3. Specificity of transport of IF-Cbl-[^{125}I]-LHRH, IF-Cbl-[^{125}I]-DP3, or IF-Cbl-Hex-[^{125}I]-DP3 over Caco-2 cell monolayers and comparison with IF-[^{57}Co]-Cbl. 200 pM solutions (0.5 ml/well) of the indicated ligands or of their complexes with IF or NIF were applied to the apical site of filter-grown Caco-2 monolayers. After 20 h at 37°C transcytosed, surface-bound, and internalized radioactivity were determined.

Table 1. Centricon-30 Analysis of Apical and Basolateral Media from Caco-2 Transcytosis Studies

IF complexed with	Source	Centricon-30 Filtrate	Centricon-30 Retentate
Cbl	Apical	1	99
	Basolateral	1	99
Cbl-DP3	Apical	4	96
	Basolateral	23	77
Cbl-Hex-DP3	Apical	2	98
	Basolateral	15	85
Cbl-LHRH	Apical	6	94
	Basolateral	52	48[(A)]
Antibody studies with Cbl-LHRH			
Radioactivity released* from the basolateral Cbl-LHRH retentate A		95[(B)]	5
Filtrate B + anti-LHRH antibody		98	2
Control:			
Basolateral medium^{+} + Cbl-LHRH		5	95[(C)]
Radioactivity released* from Cbl-LHRH retentate C + anti-LHRH antibody		9	91

* [(A)] Source of retentate A; [(B)]source of filtrate B; [(C)]source of rentate C. TCII-bound radioactivity in BL medium was released by incubation with a > 10,000-fold excess of Cbl for 12h at 37°C.
$^{+}$ Contains secreted TCII.

Oral Absorption and Tissue Distribution of Cbl and Cbl-Peptide Conjugates

Oral absorption of the radiolabeled Cbl-peptides was studied in the rat by applying the free ligands with or without a >10^5-fold excess of cold Cbl into the rat stomach by oral gavage. After 4 hrs or 24 hrs, animals were sacrificed, their intestines were extensively rinsed to eliminate adsorbed or luminal ligands and total radioactivity in the body tissues and urine was recovered and measured. As Cbl absorption is saturable, all ligands were administered under the Cbl uptake capacity of the rat intestine. Figure 4 indicates that at 1 pMol dose, the rat

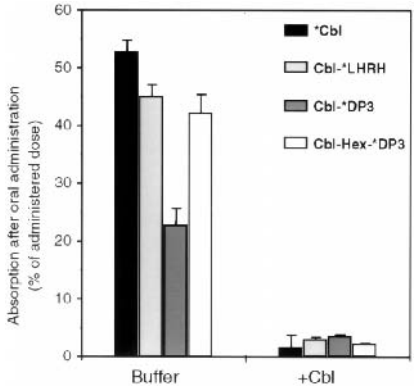

Fig. 4. Intestinal absorption of [^{57}Co]-Cbl, Cbl-[^{125}I]-LHRH, Cbl-Hex-[^{125}I]-DP3, and Cbl-[^{125}I]-DP3 in the rat. Radiolabeled ligands alone, or in the presence of Cbl, were administered to rats by oral gavage and urine and feces were collected. After 24 h, animals were sacrificed, their intestines were flushed to remove unabsorbed material and tissues were collected to measure their radioactivity. Absorption is the total radioactivity recovered in body tissues and urine divided by the total amount of radioactivity administered.

Table 2. Percent of Absorbed Radioactivity in Tissue After Oral Administration to Rats

Compound	Cbl		Cbl-LHRH		Cbl-DP3		Cbl-Hex-DP3		DP3		LHRH
Time	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h
Liver	7	7	4	2	5	8	6	7	2	1	3
Kidney	11	22	6	6	6	27	10	26	1	1	2
Small intestine	40	8	21	4	53	16	46	11	36	2	1
Muscle + Skin	29	42	48	16	19	21	26	34	36	2	37
Plasma	8	1	10	5	6	1	5	1	19	2	17
Urine	1	1	5	66	3	22	4	15	1	80	34
Other*	4	5	6	1	8	5	3	5	5	12	6
% of oral dose absorbed	46	53	40	45	19	23	30	42	4	15	32

* Other tissues: spleen, lung, heart, stomach, colon, caecum, testis, and brain.

intestine absorbs 53% of applied [^{57}Co]-Cbl and this absorption could be inhibited >20-fold by unlabeled Cbl. The same doses of Cbl-[^{125}I]-LHRH and Cbl-Hex-[^{125}I]-DP3 resulted in absorption of 45% and 42%, respectively, which also could be inhibited by an excess of unlabeled Cbl. Cbl-[^{125}I]-DP3 had a substantially lower absorption: 23% of administered radioactivity was recovered.

The tissue distribution of absorbed radioactivity after oral administration is presented in Table 2. High concentrations of radioactivity per gram tissue for [^{57}Co]-Cbl, Cbl-[^{125}I]-LHRH, Cbl-[^{125}I]-DP3 and Cbl-Hex-[^{125}I]-DP3 were detected in small intestine and kidney. Between 4 h and 24 h after administration, the concentrations of [^{57}Co]-Cbl, Cbl-[^{125}I]-DP3 and Cbl-Hex-[^{125}I]-DP3 decreased in the small intestine and increased in the kidney. Excretion of these three compounds into urine was relatively slow. In contrast, excretion of radioactivity from orally administered [^{125}I]-LHRH or [^{125}I]-DP3 into urine was high and tissue distribution of radioactivity differed significantly from their Cbl-coupled counterparts. Distribution of orally administered Cbl-[^{125}I]-LHRH in the body was similar to the other Cbl-conjugates after 4 h. However, after 24 h its distribution resembled that of the uncoupled peptides.

The comparison of oral absorption with i.v. injection of Cbl and Cbl-conjugates into cannulated rats showed that unmodified peptides were eliminated faster from plasma than Cbl-conjugates (data not shown), with >60% of the administered radioactivity excreted into urine within 24h (Table 3). In contrast, only <1% and <13% of the radioactivity of Cbl and of Cbl-DP3/Cbl-Hex-DP3 was excreted, respectively. Contrary to the Cbl-coupled peptides, [^{125}I]-DP3 and [^{125}I]-LHRH also did not accumulate in the kidney and showed low levels in the small intestine.

In order to detect absorbed material that had permeated unequivocally beyond the intestinal epithelium, [^{125}I]-containing material was extracted from rat kidney, where substantial amounts of the applied dose had accumulated 24 hrs after oral administration (Table 2). In SDS-PAGE, the starting material, Cbl-Hex-[^{125}I]-DP3, exhibited an unusual dual band pattern with apparent M_r of 18K and 16K (Fig. 5, lane 1). These two bands represent the mono- and di-iodination products of the conjugate as indicated by mass spectrometry (data not shown). In IEF, Cbl-Hex-[^{125}I]-DP3 had a pl of 3 or less (lane 1). When CD-29 was added, its position shifted to the pl of the antibody (Fig. 5, lane 2). After addition of an excess of Cbl and incubation for 30 min at 80 °C, Cbl-Hex-[^{125}I]-DP3 was displaced from the CD-29-complex and migrated to its original position (Fig. 5, lane 3). The radioactive material extracted from kidney exhibited an identical pattern; it had the same two bands in SDS-PAGE (Fig. 5, lane 2) and moved to the predicted positions in IEF before (Fig. 5, lane 5)(=kidney extract containing CD-29) and after displacement with Cbl (Fig. 5, lane 4). Thus, Cbl-Hex-[^{125}I]-DP3 and the radioactivity in the kidney extract behaved indistinguishably in both analytic systems, indicating that intact Cbl-conjugate was absorbed and stored in the kidney.

DISCUSSION

In order to avoid the use of absorption enhancers, we have examined a method of achieving selective absorption of an orally administered peptide by covalently coupling it with cobalamin (Cbl, VB12) and have demonstrated that the uptake proceeds through the specific, receptor-mediated intestinal absorption pathway of this vitamin.

Table 3. Percent of Administered Radioactivity in Tissue After Intravenous Administration to Rats

Compound	Cbl		Cbl-LHRH		Cbl-DP3		Cbl-Hex-DP3		DP3		LHRH	
Time	8 h	24 h	8 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	8 h	24 h
Liver	3	4	3	2	5	3	7	6	1	1	1	1
Kidney	26	36	5	3	14	24	16	29	1	1	1	1
Small intestine	5	5	4	27	11	8	11	8	4	1	1	1
Muscle + Skin	60	49	79	2	32	15	35	26	3	2	11	7
Plasma	2	1	1	1	3	1	4	1	2	0	1	0
Urine	1	1	6	47	4	13	2	13	48	61	66	76
Other*	3	4	2	18	31	36	25	17	37	34	18	14

* Other tissues: spleen, lung, heart, stomach, colon, caecum, testis, and brain.

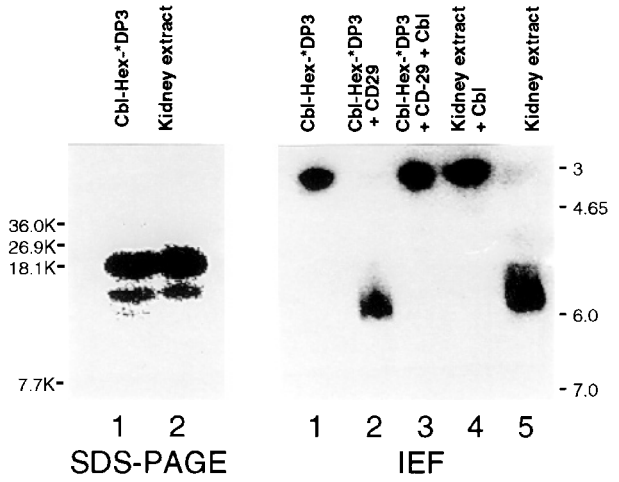

Fig. 5. Integrity of Cbl-Hex-[^{125}I]-DP3 in rat kidney after oral administration to rats. Cbl-Hex-[^{125}I]-DP3 was administered to fasted rats by oral gavage. After 24 h, rats were sacrificed and radioactivity was extracted from kidneys using the monoclonal anti-Cbl antibody CD-29. Extracts were analyzed by SDS-PAGE and IEF. Cbl-Hex-[^{125}I]-DP3 served as a reference (Lane 1, SDS-PAGE and IEF). In IEF, addition of CD-29 to Cbl-Hex-[^{125}I]-DP3 results in a shift of the latter's isoelectric point from 3 to about 5.8 (=pl of CD-29) (Lane 2). This shift in migration is reversed by addition of an excess Cbl (Lane 3). Similarly the radioactivity in the kidney extract containing CD-29 migrates with a pl of 5.8 and of 3 before (lane 5) and after displacement with Cbl (lane 4), respectively.

Two compounds were investigated: a lipid-insoluble, metabolically stable octapeptide composed of D amino acids (DP3, M_r 784) (15) and a D-Lys^6-LHRH analogue (22). Earlier studies suggested that, with the exception of substitutions at the Co-β position (cyanide in native Cbl), substitutions of the Cbl molecule compromise its recognition by IF (16,17). However, the relative binding affinities of Cbl or Cbl-LHRH for IF as measured in a radioimmunoassay (RIA) were indistinguishable, indicating that, apart from the Co-β position, the "e" position of the corrin ring can be modified without significant loss of IF affinity. The affinity of Cbl-DP3 for IF was about one order of magnitude less than that of the Cbl-LHRH, but incorporation of a hexyl spacer (Cbl-Hex-DP3) resulted in a 5-fold better recognition by IF (Fig. 1). All three ligands bound several thousand-fold better to IF than cobinamide, which is a Cbl analogue that lacks the nucleotide moiety and has low affinity for IF (23). All conjugates had comparable affinity for NIF (Fig. 1).

Recognition and transcytosis of preformed complexes of IF and Cbl or Cbl conjugates by cellular receptors (IFCR) was studied with monolayers of Caco-2 cells expressing IFCR on their apical surface (23–25). Binding of IF-complexes to IFCR was saturable in the picomolar range (data not shown) and specific, since a 20-fold excess of IF-Cbl, but not of non-intrinsic Factor-Cbl inhibited the binding of the complexes to the IFCR (Fig. 2); non-intrinsic Factor is a Cbl-binding protein lacking a cellular receptor (25,26). All IF-Cbl-conjugate complexes were specifically transcytosed across Caco-2 cell monolayers (Fig. 3). Binding, internalization and transcytosis of Cbl-LHRH and of Cbl-Hex-DP3 were comparable to Cbl. In contrast, significantly less Cbl-DP3 was transcytosed, although similar amounts of IF-Cbl-DP3 complexes bound to and were internalized by Caco-2 cells. This might be explained by the lower affinity of this conjugate for IF (Fig. 1) and/or by differences in the affinity of the Cbl-peptides for transcobalamin II (TCII). In the Caco-2 model, TCII is secreted into apical and basolateral medium and, after binding to Cbl or Cbl-peptides, these complexes bind to TCII receptors and are internalized by the cells (21,27). In support of this concept are also the relative high amounts of surface-bound and internalized radioactivity in the buffer control (Fig. 3) and our recent observation that even Cbl in IF-Cbl complexes is partially transferred to TCII in the apical compartment during Caco-2 transcytosis experiments (Alsenz *et al.*, manuscript in preparation).

Analysis of the transcytosed radioactivity revealed that only the metabolically stable Cbl-DP3 and Cbl-Hex-DP3 passed the cells intact (Table 1). In contrast, most of the transcytosed Cbl-LHRH seems to represent degraded material since (i) more than 50% of the basolateral radioactivity passed a 30'000 cut-off membrane and (ii) material released from Cbl-binding proteins was not recognized by an anti-LHRH antibody (Table 1). Similar results were obtained in gelfiltration studies or when basolateral medium was subjected to TCA precipitation (data not shown). Since Cbl-LHRH added to the AP or BL side of cells for 24h was not cleaved, degradation seems to occur inside the cells (data not shown).

In vivo, after administration of equal doses of radiolabeled compounds to rats by oral gavage, 53%, 45%, 42% and 23% of the radioactivity of Cbl, Cbl-LHRH, Cbl-Hex-DP3 and Cbl-DP3, respectively, was absorbed from the intestine (Fig. 4). The extent of absorption of the compounds *in vivo* correlated well with their *in vitro* affinity for IF (Fig. 1) and with the transcytosis studies in the Caco-2 model (Fig. 3). Simultaneous administration of a $>10^5$-fold excess of unlabeled Cbl reduced uptake of all compounds to $<4\%$, indicating that the uptake was specific and occurred through the Cbl pathway. Cbl had no effect on the uptake of ^{125}I]-DP3 and [^{125}I]-LHRH (data not shown). The relatively high uptake of the latter two can be explained by their lower MW (paracellular diffusion) and, in case of LHRH, by the uptake of labeled degradation products (Table 2).

The pharmacokinetics of orally administered Cbl and of the metabolically stable Cbl conjugates were very similar, with plasma radioactivity peaks after about 4 h (data not shown) and the highest tissue concentration in small intestine with almost no urinary excretion of radioactivity after 4 h (Table 2). Between 4 h and 24 h, most of the radioactivity disappeared from the small intestine and high concentrations were localized in the kidney and liver with little urinary excretion. Sequestration of Cbl in the kidney is well established and recently, megalin was reported to mediate uptake of TCII-Cbl complexes from the luminal side (28). It indicates that the absorbed radioactivity is still associated with the Cbl moiety. This is further supported by the different distribution of the uncoupled DP3 and LHRH peptides, which do not accumulate at all in the kidney and are rapidly excreted into urine (Table 2 and 3).

The distribution pattern of Cbl-LHRH suggests a partial or almost complete degradation of the conjugate during absorption and/or distribution. The initially high levels in the small intestine after oral administration decrease rapidly but radioactivity does not accumulate in the kidney and is excreted into urine similar to the uncoupled peptides (Table 2). This degradation is not necessarily a consequence of the intestinal absorption

step alone, as suggested by the Caco-2 transcytosis results (Table 1). Although Cbl-LHRH itself is not metabolically stable, intact Cbl-LHRH has been identified in solubilized rat intestinal enterocytes, after oral administration of Cbl-LHRH and in intestinal juice after pre-complexation with IF (P.C. de Smidt *et al.*, manuscript in preparation). Therefore, complexation to IF seems to inhibit proteolytic degradation of Cbl-LHRH as has been suggested for Cbl-GCSF in a previous study (18). Once in the circulation, Cbl-LHRH seems to be further subjected to degradation as indicated by the rapid excretion of Cbl-LHRH after intravenous injection when compared to the metabolically stable Cbl-conjugates (Table 3). Nonetheless, some pharmacological active peptide seems to reach the circulation, since pharmacodynamic studies in mice with Cbl-LHRH and in rats with Cbl-GCSF have documented effects of these compounds, although the extent and mechanism of uptake were not analyzed (18,22).

In contrast to Cbl-LHRH, radiolabeled Cbl-Hex-DP3 recovered from kidney homogenates was indistinguishable from the original Cbl-Hex-[^{125}I]-DP3 as assessed by its isoelectric point, its binding by a monoclonal anti-cobalamin antibody (CD-29), and its unusual electrophoretic behavior in SDS-polyacrylamide gel electrophoresis (see Material and Methods section) The [^{125}I]-labeled Cbl-Hex-DP3 exhibits two bands with M_r of 16K and 18K which represent the mono-and di-iodinated molecules, as already mentioned) (Fig. 5).

It will now be important to identify the size limit and the maximum amount of efficacious peptide that can be delivered to the circulation via the Cbl pathway. In addition, appropriate biodegradable linkers have to be identified that allow the release of active polypeptide from Cbl into blood after intestinal absorption. Preliminary *in vitro* experiments indicate that Cbl-peptide conjugates up to 20 K possess biological recognition, since Caco-2 cells took up IF-Cbl-interferon alpha complexes (J. Alsenz and P. C. de Smidt, unpublished data). Since Cbl uptake in humans is limited to around 1–2 μg per dosage (12,29) (for a larger peptide-conjugate such as Cbl-interferon alpha this amounts to 20 μg), the amount of peptide that can be introduced through the Cbl pathway is limited. This limitation could be counteracted by multiple dosing (IFCR recycles every 30 minutes (20)) or by the utilization of Cbl-tagged particulate carriers (30) that could simultaneously protect their contents from proteolytic degradation.

The current investigations have presented evidence that utilization of the Cbl pathway can be a realistic option for oral peptide delivery and have documented the highest level of oral peptide absorption under physiological conditions yet reported.

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