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SYNTHESIS AND SECRETION OF COBALAMIN BINDING PROTEINS BY OPOSSUM KIDNEY CELLS

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Opossum kidney epithelial cells synthesize and secrete two Cobalamin (Cbl) binding proteins of Mr 66,000 and 43,000. When grown on culture inserts, the apical medium contained both these proteins while the basolateral medium contained only the 43 kDa Cbl binder. Colchicine, a microtubule disruptive drug, increased two fold the apical but not the basolateral secretion of the Cbl binding proteins. Although the opossum Cbl binders did not cross react with antiserum raised to Cbl binders from other species, the identity based on Cbl binding and size suggest that the 66 kDa and 43 kDa proteins are haptocorrin and transcobalamin II.

It has been recognized for some time that the kidney plays an important role in maintaining plasma levels of Cobalamin (Cbl; Vitamin B_{12}) (1,2). The kidney seems to accomplish the regulation of Cbl levels by sorting Cbl during periods of loading and by releasing its reserves to other tissues during Cbl deficiency (3-6). Cobalamin entry into the renal proximal tubular cells is mediated by two uptake systems. One, via transcobalamin II (TC II) (2) and the other via intrinsic factor (IF) (7). Available evidence (8) suggests that following the delivery of Cbl into the cells these two protein ligands are degraded by the lysosomes. However, the mechanism by which Cbl stored (1,2) in the kidney is sorted bound to Cbl binders to be either excreted in the urine or recirculated to the tissues is not known. The current studies were undertaken to address the issues of synthesis and direction of secretion of Cbl binding proteins in polarized renal proximal tubular cells. In this report, we use metabolic labeling of OK cells, a stable cell line from opossum kidney epithelial cells, to study these processes. We show that TC II and haptocorrin, two Cbl binding proteins, are synthesized by OK cells. Furthermore, our studies also show that while TC II is secreted bidirectionally, haptocorrin secretion occurs only via the apical domain. Colchicine, a microtubule disruptive drug increases the apical but not the basolateral secretion of these Cbl binding proteins.

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MATERIAL AND METHODS

The following chemicals were purchased commercially as indicated: [^{57}Co]cyanocobalamin (15-220 μCi/μg) (Amersham Corporation, Arlington Heights, IL), Trans[^{35}S]label (> 1000 Ci/mmol containing ≥ 70 % methionine, ≤ 15% cysteine, ≤ 7% methionine sulfone, ≤ 3% cysteic acid and ≤ 5% other [^{35}S] compounds (ICN Radiochemicals, Irvine, CA), QUSO G-32 (Philadelphia Quartz Company, Valley Forge, PA) Colchicine and Sephadex G-150 (Sigma Chemical Co., St. Louis, MO). Haptocorrin from porcine stomach was purified and anti-serum was raised in rabbits as described earlier (9). Anti-serum to milk haptocorrin, hog IF and rabbit TC II were a generous gift from Dr. Robert H. Allen (University of Colorado Health Science Center, Denver, CO). Anti-serum to pure human TC II (gift from Dr. Charles Hall, VA Medical Center, Albany, NY) was raised in rabbits as described earlier (9).

OK cells were obtained from Dr. Beatrice States (Childrens Hospital, Philadelphia, PA) and were used between passage 75-95. The cells were grown on T-75 flasks or 30 mm diameter tissue culture inserts as described earlier (7). Cells grown on filters for 6-8 days had transepithelial resistance between 50-60 Ohms/cm^2 and were used for metabolic labeling and secretion studies. OK cells grown for 6 days on either T-75 flasks or culture inserts were incubated for 15 min. at 37^0 C in methionine free medium. Trans[^{35}S]-label (50 μCi/ml) was added and the cells were incubated for 12 h at 37^0 C. The labeled medium was dialyzed against 6 L of 5 mM Tris-HCl pH 7.4 containing 0.1 mM PMSF and 2 mM Benzamidine. The dialyzed medium (10 ml) from labeled monolayers from one T-75 flask or 4 culture inserts were passed on separate Cbl-sepharose columns (10 x 1 cm). The columns were washed exhaustively with (a) 1 L of 10 mM K-PO_4 buffer pH 7.4 containing 140 mM NaCl and 0.1 mM PMSF, (b) 500 ml of the same buffer containing 0.1 M guanidine-HCl. The bound radioactivity was eluted with the same buffer containing 5 M guanidine-HCl. The eluate was dialyzed for 48 h against 12 L of 1 mM K-PO_4 pH 7.4 containing 0.1 mM PMSF and 2 mM Benzamidine with a 4 L change after every 16 h. The dialyzed radioactivity was concentrated 20 fold and used for SDS electrophoresis (7.5%) according to Lammeli (10) and subjected to autoradiography. The effect of colchicine on the secretion was carried out essentially according to Rindler et al (11) as described earlier (7). The binding of [^{57}Co] Cbl by the medium was carried out using albumin coated charcoal method of Gottlieb et al (12). When the cells were grown on T-75 flasks for 3-15 days, fresh media without fetal calf serum was added 12 h before the activity measurement for each growth period.

The affinity purified [^{35}S] labeled proteins (10-15000 cpm) obtained from monolayers were treated with QUSO G-32 (5 mg). The pelleted resin was washed once with Tris buffered saline and the radioactivity was released by boiling with SDS (1%).

[^{57}Co]Cbl (0.37 pmol) bound to purified hog haptocorrin and opossum haptocorrin obtained from Sephadex G-150 column or freshly prepared serum (500 μl) was treated with 15 μl of anti-serum to various Cbl binding proteins and incubated for 12 h at 5^0 C. Formalin fixed staph A cell suspension (Gibco BRL, Grand Island, NY) (125 μl) was added and the reaction mixture was continuously agitated for 4 h at 5^0 C. The reaction mixture was centrifuged at 10,000 xg for 15 min. The radioactivity in the supernatant and particulate fraction were determined using Beckman Gamma P 4000 counter.

The media collected from cells that were grown for 6 days in T-75 flasks were allowed to bind [^{57}Co]Cbl. The protein bound radioactivity was concentrated and loaded on to a Sephadex G-150 column (1.25 x 90 cm) and developed with 10 mM Tris-HCl pH 7.4 at a flow rate of 10 ml/h. One hundred fractions of 1 ml size were collected and counted.

RESULTS

The total [^{57}Co]Cbl binding activity in the media rose about 10-15% from day 3 to 6, but then onward the binding activity declined and by day 15 about 90% of the maximum activity was still observed (Figure 1a). When [^{57}Co]Cbl complexed to the media from cells grown for 6 days was

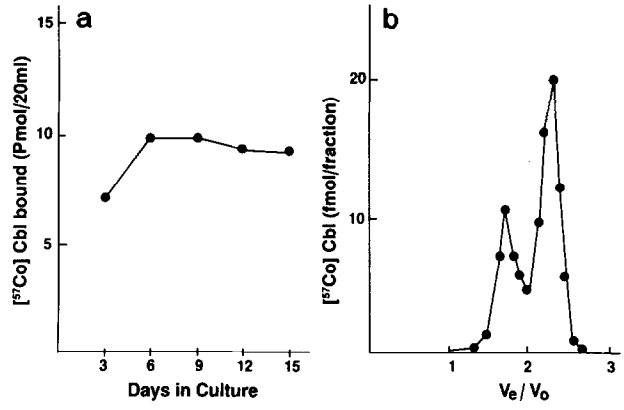

Figure 1. a) <u>Expression of [^{57}Co] Cbl binding activity in the medium.</u>
OK cells plated at a density of 1.5 X10^6 cells were grown in 75-cm^2 flasks. Fresh medium was added 12 h before the cells were harvested at indicated time intervals. [^{57}Co] Cbl binding was determined using 1 ml of the medium. The values reported are duplicate assays performed by using medium from cells of three different passages.
b) <u>Gel filtration of protein bound [^{57}Co]Cbl complex from the medium.</u>
[^{57}Co] Cbl complex to the medium from cells grown for 6 days was fractionated on Sephadex G-150. Other details are provided in methods.

separated on Sephadex G-150 two radioactive peaks (Figure 1b) were observed. The apparent Mr of the two peaks were around 70 kDa and 45 kDa. In order to confirm that the OK cells synthesized and secreted two Cbl binding proteins, the cells were metabolically labeled with trans[^{35}S]-label. The labeled media after purification on Cbl-sepharose affinity columns demonstrated on SDS- PAGE two proteins of Mr 66 kDa and 43 kDa (Figure 2, lane 2). Bulk of the 43 kDa and very little of the 66 kDa protein was precipitated with QUSO G-32 (Figure 2, lane 3), a resin known to specifically precipitate TC II (13). Further confirmation of the 43 kDa protein as TC II and the 66 kDa protein as haptocorrin was tested by treatment of the purified [^{35}S]-labeled proteins with anti-serum to human or rabbit TC II or hog stomach haptocorrin. The 43 kDa protein was present in the immune supernatant when treated with either rabbit TC II (Figure 3, lane 5) or human TC II (Figure 3, lane 6) anti-serum. The 66 kDa protein was similarly present in the supernatant (Figure 3, lane 7) following treatment with anti-serum to hog haptocorrin. The immune pellets obtained using rabbit TC II (Figure 3, lane 2) or human TC II (Figure 3, lane 3) and hog haptocorrin (Figure 3, lane 4) anti-serum did not demonstrate any detectable 43 kDa or 66 kDa protein, both of which were present in the [^{35}S]labeled fraction used (Figure 3, lane 1) for immunoprecipitation. Furthermore, radioactive Cbl complexed to opossum haptocorrin or serum could not be precipitated with anti-serum to haptocorrin from hog and milk or rabbit or human TC II respectively. These anti-sera were able to precipitate > 90-95% of [^{57}Co]Cbl complexed to the respective Cbl binders from the same species (Table 1).

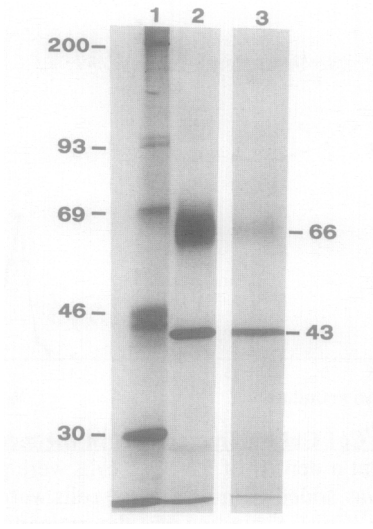

Figure 2. **SDS-PAGE of [^{35}S]labeled Cbl binders.**
[^{35}S] labeled proteins from culture media of cells grown for 6 days were purified on Cbl-Sepharose. The purified proteins subjected to SDS-PAGE on 7.5% gels. Lane 1. [^{14}C] markers. Lane 2. Affinity purified [^{35}S] proteins. Lane 3. [^{35}S]-protein precipitated with QUSO G-32.

When the OK cells were cultured on filters, Cbl binding activity was secreted in both the apical and basolateral directions (Figure 4). However, specific activity of Cbl binding in the apical medium was nearly double of the activity secreted into the basolateral medium. The identity of two Cbl binders in the apical and one Cbl binder in the basolateral medium was further confirmed by the SDS-gel electrophoresis of [^{35}S]labeled proteins purified using Cbl-sepharose affinity columns. The apical medium again demonstrated two proteins of Mr of 66 kDa and 43 kDa, while the basolateral medium contained only the 43 kDa species (Figure 5).

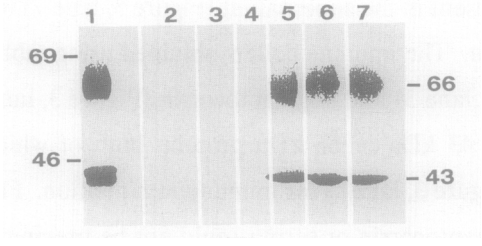

Figure 3. **SDS-PAGE of immuno-precipitated [^{35}S]-Cbl binders.**
Lane 1. The relative position of Cbl binders prior to addition of anti-serum. Lanes 2,3,4 and 5,6,7 represent the radioactivity present in immuno-particluate and supernatant fractions respectively, using anti-serum to rabbit TC II (lanes 2 and 5), human TC II (lanes 3 and 6) and hog haptocorrin (lanes 4 and 7).

Table 1

Immunoprecipitation of [^{57}Co] Cbl Complexed to Haptocorrin and Plasma

	HAPTOCORRIN		SERUM		
	Hog	Opossum	Human	Rabbit	Oppossum
	Precipitated (%)				
Anti-serum to:					
Haptocorrin					
Hog Stomach	95 ± 4	10 ± 2	0	0	0
Human Milk	90 ± 5	0	0	0	0
Transcobalamin II					
Human	0	0	95 ± 4	0	0
Rabbit	0	0	0	94 ± 3	0

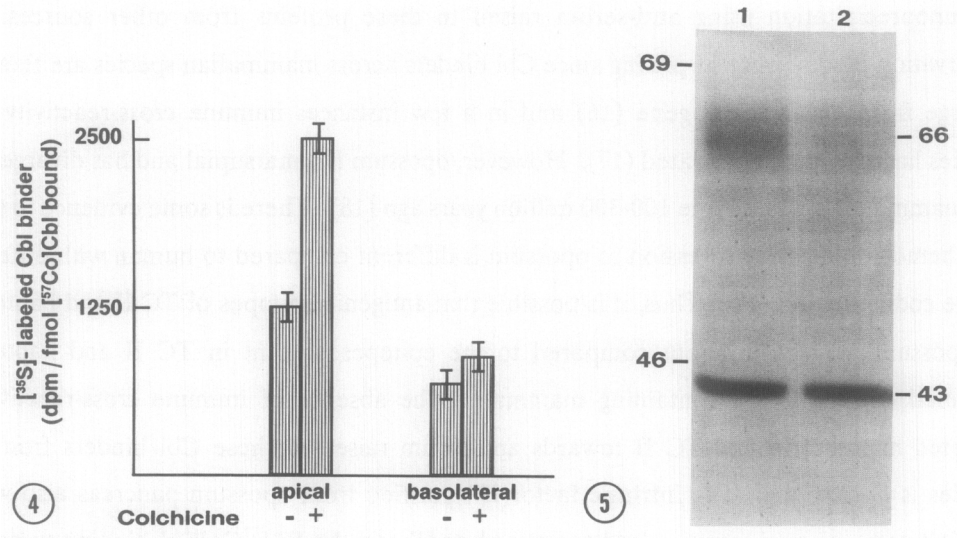

Figure 4. Direction of secretion and the effect of Colchicine.
OK cells grown on culture inserts were labeled with Trans [^{35}S] label for 12 h in the presence and absence of Colchicine (20 µg/ml apical and 40 µg/ml basolateral). Both the [^{57}Co] Cbl binding and the amount of [^{35}S] proteins bound to Cbl-sepharose were determined. The specific activity (cpm/fmol Cbl binding) is shown and is a mean ± SD two separate labeling experiments using 6 filters each time.

Figure 5. SDS-PAGE of [^{35}S]-Cbl binders from the apical and basolateral medium.
The [^{35}S]-radioactivity in the apical and basolateral media was passed over on Cbl-sepharose culumns separately. The eluted radioactivity was dialyzed, concentrated and used for electrophoresis. Lane 1. Apical medium. Lane 2. Basolateral medium.

Colchicine increased only the apical secretion of [^{57}Co]Cbl binding (Figure 4), or the amount of [^{35}S] labeled Cbl binders secreted apically (Figure 4).

DISCUSSION

In this study we have examined the synthesis and secretion of Cbl binding proteins in a proximal tubular cell line, OK. Our results show for the first time the de novo synthesis and secretion two Cbl binding proteins of Mr 66 kDa and 43 kDa. These two proteins represent haptocorrin and TC II, respectively. This conclusion is based on the following evidences. First, the two proteins were purified from affinity chromatography columns using Cbl-sepharose as an affinity ligand. Second, the Mr of these two proteins exactly coincided with the reported values for haptocorrin (14) and TC II (15). Third, bulk of the 43 kDa protein was selectively precipitated with QUSO G-32, a microfine precipitate of silica that selectively absorbs TC II, but not TC I or TC III, the haptocorrins present in plasma (13). Finally, the gel filtration profile of [^{57}Co]Cbl revealed two [^{57}Co]Cbl bound peaks of apparent Mr of 70,000 and 45,000.

Although the above mentioned evidences strongly suggest the identity of these two Cbl binders synthesized in OK cells as haptocorrin and TC II, we were unable to confirm this by immunoprecipitation using anti-serum raised to these proteins from other sources. This observation is somewhat surprising since Cbl binders across mammalian species are thought to emerge from an ancestral gene (16) and in a few instances immune cross-reactivity across species have been demonstrated (17). However, opossum is a marsupial and has diverged from the mammalian species some 100-300 million years ago (18). There is some evidence to suggest that hemoglobin gene expression in opossum is different compared to human with differences in the coding region (19). Thus, it is possible that antigenic epitopes of TC II and haptocorrin in opossum may be different compared to the epitopes present in TC II and haptocorrin obtained from placenta containing mammals. The absence of immune cross-reactivity for secreted haptocorrin and TC II towards anti-serum raised to these Cbl binders from other species is not unique. The intrinsic factor(IF) purified from opossum pancreas also was not precipitated with anti-serum raised to rat or hog IF and the IF-[^{57}Co]Cbl binding to opossum renal receptor cannot be blocked with anti-serum to rat or dog IF-Cbl receptor (B. Seetharam et al, unpublished results). Furthermore, opossum mannose 6-phosphate/IGF II receptor appear to be different from the bovine liver receptor both kinetically and immunologically (N Dahms, personal communication). Thus, it is likely that more than one protein expressed in opossum is structually different.

The direction of secretion of TC II by OK cells is rather unique in that roughly equa amounts were secreted in both the apical and basolateral direction. Another polarized cell, the

human adenocarcinoma (CaCo-2) cells synthesize only TC II and secrete it exclusively only via the basolateral domain (20). The bidirectional secretion of TC II by the OK cells in the current study and the unidirectional secretion of TC II by the CaCo-2 cells (20) occurred in the absence of entry of Cbl into these cells, suggesting that TC II undergoes constitutive secretion in these polarized cells. Although the sorting mechanism for TC II is not clear, it is possible that no receptors are involved in sorting and hence TC II would partition with the fluid phase into both the apical and the basolateral vesicles. Haptocorrin on the other hand is secreted unidirectionally via the apical domain. The sorting signals responsible for the differences noted with the direction of secretion of TC II and haptocorrin by OK cells are not clear at the moment. However, it appears from earlier studies with leukocytes (21,22) that the secretion of haptocorrin may be regulated. The effect of microtubule disruption on the increased apical secretion of Cbl binders may be due to altered kinetics of secretion caused by disruption in the interactions between the apical destined secretory vesicles and the microtubular network. Earlier studies (7,23,24) have shown that nocodazole, another microtubule disruptive drug, decreased the apical secretion or the apical membrane insertion of proteins in CaCO-2 and OK cells. Thus, the increased secretion of Cbl binders in the OK cells due to treatment with colchicine may be unique to Cbl binders. Further work is needed to clarify the consequence of microtubule disruption on polarized movement of secretory proteins in various polarized epithelial cells. In conclusion, the resuts of the present study show that OK cells secrete immunologically different forms of Cbl binding proteins. The direction of secretion of these proteins suggest that TC II may mediate the exit of Cbl both for excretion (apical) and for tissue reutilization (basolateral). The haptocorrin due to its ability to bind Cbl analogues with high affinity (25) may mediate the excretion of harmful Cbl analogues. Further studies are needed to test this hypothesis.

Acknowledgments

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